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BIOSYNTHESIS OF COENZYME A BY TISSUE HOMOGENATES

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Coenzyme A (CoA) discovered by Lipmann, as a derivative of pantothenic acid, is active in fatty acid and stearin biosynthesis and is necessary for the acetylation of aromatic amines, acetic and oxaloacetic acids, hexosamines and histamines. Due to the presence of a sulfhydryl group in CoA the transfer of the acetyl group is accomplished by the formation of an intermediate labile compound: acetyl CoA. CoA carries out a similar transfer of other acid radicals such as succinyl, propionyl, butyryl, benzoyl, etc., i.e., acyls. Thus, pantothenic acid participates in the anabolic processes of the organism in the form of CoA [1]. Of significant interest is the process of conversion in the organism of the pantothenic acid found in food into CoA.

In a previous report [2] on the biosynthesis of CoA we demonstrated the ability of rat and guinea pig brain homogenates to synthesize in vitro CoA from pantothenate in the presence of adenosinetriphosphoric acid (ATP), cysteine and Mg^{++}. The successful synthesis of CoA in purified fresh extract of pigeon liver in the presence of the same ingredients was reported in later works [3-5]. These authors showed that the biosynthesis of CoA from pantothenate occurs in a number of stages. The pantothenate reacts with cysteine to form pantetheine, which in turn is phosphorylated to 4'-phosphopantetheine in the presence of ATP; the latter reacts with another molecule of ATP to form the Coenzyme A pyrophosphate, which in turn is phosphorylated to CoA in the presence of ATP.

However, the intensity of CoA biosynthesis in vitro in different animal organs and the influence of various factors on this process have been insufficiently investigated at the present time. We studied the biosynthesis of CoA in various animal organs and have found that of all the tissues examined the brain homogenates were synthesizing CoA most intensively. The influence of thiamine and pyruvate on CoA synthesis was investigated. The pyruvate forms an acetyl which combines with CoA in the presence of thiamine. Since the most intensive synthesis of CoA occurs in the brain, we carried out all subsequent experiments on brain homogenates. We studied the biosynthesis of CoA in brain homogenates under such pathological conditions as pantothenic deficiency in rats, in human tumors, and upon the irradiation of guinea pigs by x-rays.

EXPERIMENTAL

Methods

Albino rats weighing approximately 150 g and guinea pigs weighing approximately 300 g were decapitated and a tissue sample was immediately homogenized for 3-4 minutes with cooling. Each g of tissue was homogenized in 6 ml of liquid containing 2.5 ml of 0.1 M glycine buffer (pH 7.4), 0.76 mg of $MgCl_2$, 4.84 mg of cysteine, 10 mg of ATP and 1 mg of calcium pantothenate dissolved in the remaining 3.5 ml of water. The obtained homogenate was divided into 4 equal samples, one of which, the control, was immediately inactivated by a 3 minute heating on a boiling water bath, while the remaining 3 samples were incubated in a water thermostat with agitation at 30° for 30, 60 and 90 minutes, and subsequently inactivated. In experiments involving the addition of thiamine and pyruvate the latter were added in the amount of 1 and 10 mg respectively per 1 g of tissue.

In experiments involving pantothenic deficiency we used rats weighing approximately 40 g, separated them from their mother and kept them for 5-9 weeks on a synthetic pantothenic-acid-free casein sugar diet (experimental group) or on the same diet with the addition of 2 mg of calcium pantothenate per 100 g of

diet (control group). The composition of the diet was taken from the work of Olson and Kaplan [6] and the composition of the mineral salts from the work of Hegsted and Miles [7]. Casein was made vitamin-free according to the procedure of Maslennikova [8]. The above synthetic diet to which vitamins (except pantothenate) and other components were added regained its full value upon the addition of pantothenic acid, as is apparent from the weight-growth curves of Fig. 1.

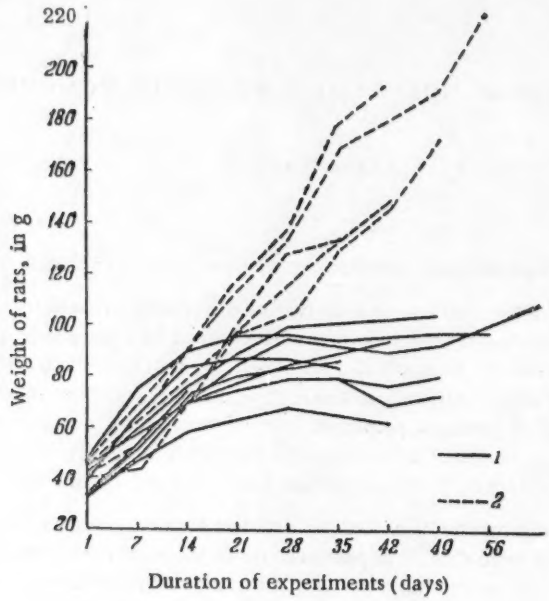

Fig. 1. Growth curves of rats:

1) Pantothenic-acid-free diet; 2) full diet.

Determination of CoA

CoA was determined by the method of Handschumacher [9]. Instead of the apoenzyme obtained from pigeon liver, we used successfully the apoenzyme obtained from chick liver. The liver of a freshly killed chick was removed and immediately frozen in dry ice. After remaining for 24 hours in the frozen state the liver was homogenized with 20 times its volume of pure dry acetone for three minutes in a cooled homogenizer. The homogenate was immediately pipetted off and the residue was washed with acetone and ether (peroxide-free). The powder was quickly dried in a vacuum desiccator over calcium chloride at a temperature lower that 0° and was preserved in a refrigerator. The extract was prepared from the acetone powder by mixing 1 g of powder with 10 ml of a 0.02 M cooled solution of $NaHCO_3$, freezing for one hour, and subsequent centrifugation. The pinkish slightly opalescent centrifugate was used as the source of apoenzyme. The extract may be preserved for a month in the frozen state. Such an apoenzyme is practically CoA-free, and is quite active upon addition of the latter.

The above method of CoA determination is based on the acetylation of the 4-aminoazobenzene substrate. The latter, when in trichloracetic alcohol solution, absorbs intensively in the 497 mμ band. As the acetylation increases, the absorption in this band decreases, and at total acetylation the absorption is equal to only 5.7% of that of the initial compound. Since pure CoA was unavailable to us at the time of the experiment we were unable to determine a standard calibration curve for the content of CoA in mg as a function of the decrease of the absorption intensity in the 497 mμ band. For this reason, we evaluated the biosynthesis of CoA by the relative ability of the inactivated homogenates, before and after incubation with the additives, to increase the acetylation of 4-aminoazobenzene in the presence of apoenzyme. The acetylation is measured by the decrease in absorption of the 4-aminoazobenzene solution in the 497 mμ band.

EXPERIMENTAL RESULTS

The Influence of Pyruvate and Thiamine on CoA Synthesis in vitro

We determined from previous experiments that the presence of ATP, cysteine, pantothenate and Mg^{++} in the incubated mixture is indispensable for the biosynthesis of CoA in brain homogenates. It has since been shown that the same ingredients are necessary for the biosynthesis of CoA by purified extracts of pigeon liver [3-5]. We performed a number of experiments to study the influence of pyruvate and thiamine on the biosynthesis of CoA in rat and guinea pig brain and liver homogenates. The results are given in Table 1.

TABLE 1

Influence of Pyruvate and Thiamine on CoA Biosynthesis in Rat and Guinea Pig Brain and Liver Homogenates

Expt. No.	Animal	Acetylation of 4-aminoazobenzene during the investigation of inactivated homogenates containing the apoenzyme (%)					
		Brain homogenate				Liver homogenate	
		With ATP	With ATP + pyruvate	With ATP + thiamine	With ATP + thiamine + pyruvate	With ATP	With ATP + pyruvate + thiamine
1	Rat	44	–	–	92	0	12
2	"	45	59	–	102	8	8.3
3	"	39	–	77	266		
4	"	206	–	206	240	0	5
5	"	56	56	–	204	30	34
6	"	50	–	–	79		
7	"	58	–	–	190		
8	Guinea pig	84	–	–	145		
9	"	40	–	44	87	10	14
10	"	27	–	–	34		
11	"	53	–	–	90		

From the data of Table 1 it is apparent that the separate addition of pyruvate and thiamine does not affect the biosynthesis of CoA while their simultaneous addition stimulates it. This stimulation is especially apparent in brain homogenates. The stimulating effect of thiamine in the presence of pyruvate indicates that thiamine, converting to its coensyme form [10], catalyzes the oxidation of pyruvate to acetate. The latter immediately forms acetyl CoA in the presence of the coenzyme.

Biosynthesis of CoA in Homogenates of Various Rat Organs

We investigated the biosynthesis of CoA in brain, liver, kidney and calf and heart muscle tissue homogenates. The curves of CoA biosynthesis in the homogenates of the above mentioned tissues are presented in Fig. 2. As can be seen from these curves, the biosynthesis of CoA is fairly intensive only in brain homogenates. Homogenates of heart muscle, kidney and liver exhibit practically no synthesis of CoA. Although a small synthesis of CoA in liver homogenates was observed in some cases in previous experiments in the present investigation, only its absence could be ascertained. This may be explained, as already noted by American authors, by the predominance of CoA hydrolysis over its synthesis in crude liver homogenates.

Biosynthesis of CoA in Brain Homogenates of Healthy Rats and of Rats with Pantothenic Acid Deficiency

Pantothenic acid is indispensable to animals for the formation of CoA and enters the organism with food. This was demonstrated in animals with pantothenic acid deficiency by the decrease in their acetylation abilities resulting from a decrease in their CoA content. The following question may then be asked: will the brain of a pantothenic acid-deficient rat, i. e., with a lower CoA content, attempt to normalize this content by a more active biosynthesis of CoA during incubation with pantothenic acid? Table 2 presents the data on CoA biosynthesis in brain homogenates of control rats and rats with pantothenic acid deficiency after 60 minutes incubation.

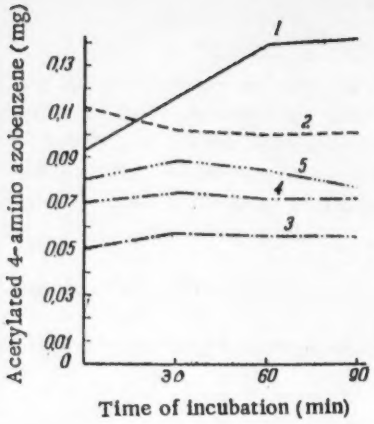

Fig. 2. Curves of CoA biosynthesis in homogenates of various organs of the rat.

1) Brain; 2) liver; 3) muscle; 4) heart; 5) kidney.

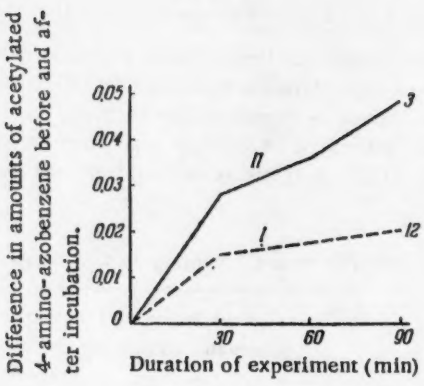

Fig. 3. CoA biosynthesis in brain homogenates of X-irradiated and non-irradiated guinea pigs.

I) Irradiated guinea pigs (average of 7 experiments); II) non-irradiated guinea pigs (average of 7 experiments).

TABLE 2

Biosynthesis of CoA in Healthy and Pantothenic Acid-Deficient Rat Brain Homogenates, After a 60 Minute Incubation with Pantothenate

Rat No.	Duration of experiment (days)	Weight of rat (g) on day of decapitation	Amount of synthesized CoA (%) expressed in acetylation of 4-amino-azobenzene
		Full diet	
1	56	222	24.6
2	48	210	16.0
3	37	168	25.0
4	49	204	47.7
5	50	206	50.0
6	58	235	30.0
			An average of 32.2% was synthesized
		Pantothenate-free diet	
7	47	74	30.0
8	53	82	41.7
9	63	107	86.0
10	43	104	36.1
11	50	66	33.5
12	61	99	59.5
13	48	86	62.9
14	70	72	325.0
			An average of 84.3% was synthesized

It becomes apparent from the data of Table 2 that the brain homogenates of rats with pantothenic acid deficiency synthesize CoA somewhat more intensively than do the brain homogenates of healthy rats. This is essentially the result of a lower initial CoA content in rat brains with pantothenic acid deficiency. However,

according to our data, the content of CoA in brains of such rats with pantothenic acid deficiency decreases little in comparison with the greater decrease in the content of flavinadenine dinucleotide or pyridoxol phosphate in the case of the corresponding riboflavin or pyridoxine deficiencies. Very low initial CoA content in the brain was noted in only one case (No. 14) out of the 8 investigated rats. This rat was kept the longest time on a vitamin-free diet, and its loss of weight was the greatest. For this reason it gave a relatively high increase in CoA during incubation with pantothenate.

CoA Biosynthesis in Human Brain Tumors

We investigated three groups of human brain tumors: the benign tumors (astrocytomas, arachnoendotheliomas), the intermediate forms (astrocytomas with differentiation or malignization phenomena, ependymomas with malignazation phenomena) and the malignant tumors (medulloblastomas, multiformous spongioblastomas, and sarcomas). The biosynthesis of CoA was investigated in homogenates of tumor tissues removed immediately after the operation. The biosynthesis of CoA in various tumors is given in Table 3.

TABLE 3

CoA Biosynthesis in Various Tumor Homogenates

No.	Family and case history number	Tumor diagnosis	Tumor character	Biosynthesis of CoA, expressed as difference between the amount of acetylated 4-aminoazobenzene before incubation and after the following periods of incubation:		
				30 min.	60 min.	90 min.
1	M – 16390	Astrocytoma	Benign	0.035	0.040	0.047
2	M – 16424	Astrocytoma	"	0.016	0.021	0.041
3	T – 16513	Ependymoblastic medulloblastoma	Malignant	0	0	0
4	M – 16442	Astrocytoma with malignization	Intermediate	0.030	0.018	0.030
5	G – 16640	Medulloblastoma	Malignant	0	0	0.013
6	K – 16464	Differentiating astrocytoma	Intermediate	0	0.014	0.016
7	P – 16585	Sarcoma	Malignant	0.011	0	0
8	G – 16683	Ependymoma with malignizations	Intermediate	0	0.004	0.004
9	L – 16704	Arachnoidendothelioma	Benign	0.020	0.042	0.018
10	E – 16815	Spongioblastic medulloblastoma	Malignant	0	0.005	0
11	K – 16806	Differentiating astrocytoma	Intermediate, close to malignant	0.017	0.012	0.002
12	P – 16908	Spongioblastoma polare, becoming multiformous	Intermediate, close to malignant	0	0.008	0.003

The most intensive biosynthesis of CoA was observed in homogenates of benign tumors, where the figures of 64, 130 and 220% were obtained. The synthesis was significantly lower in intermediate tumor homogenates, where its intensity varied from 23 to 50% and was almost completely absent from malignant tumor homogenates. Therefore, the malignant factor has a significant inhibitory action on the biosynthesis of CoA. This fact gives new support to the view that the metabolic processes in malignant tumors differ strongly from the processes in healthy tissues and benign tumors. The metabolism of benign tumors resembles more the metabolism of healthy tissues than that of malignant tumors.

Influence of x-Rays on CoA Biosynthesis

Since the use of x-ray therapy continuously expands and since a detrimental action of high x-ray doses has been established in a number of exchange processes, we decided to investigate their influence on CoA biosynthesis in guinea pig brain homogenates. Young guinea pigs weighing approximately 300 g received a total

dose of 500 r. during 10 min. After 5 days they were killed and CoA biosynthesis was determined in homogenates of their brains. On Fig. 3 are presented comparative curves of CoA biosynthesis in brain homogenates of irradiated guinea pigs, and in corresponding controls.

The data in Fig. 3 clearly show that the x-irradiation in the above doses strongly decreases the biosynthesis of CoA in guinea pig brain homogenates (in vitro). This fact confirms the view that high x-ray doses prevent the formation of substances indispensible for life, and thereby suppress the normal exchange processes.

SUMMARY

By measuring the acetylation of 4-aminoazobenzene, the biosynthesis of CoA was demonstrated in brain homogenates in the presence of pantothenate, adenosinetriphosphate, cysteine and magnesium ions. Of all the tissues studied (brain, liver, kidneys and muscle) only in the brain could synthesis of CoA be demonstrated.

Simultaneous addition of thiamine and pyruvate to the brain homogenate increases the biosynthesis of CoA, while the addition of each of them separately has no effect. Homogenates of rats with pantothenic acid deficiency synthesize CoA when incubated with pantothenate more intensively than do homogenates of normal rats.

Biosynthesis of CoA in homogenates of benign human cerebral tumors is much more intense than in malignant tumors. In cerebral tumors of the intermediate type (astrocytomas with malignization and dedifferentiation phenomena) the biosynthesis of CoA occupies an intermediate position between the two mentioned above. Biosynthesis of CoA in brain homogenates of guinea pigs is greatly reduced after exposure to total 10 minute irradiation with Roentgen rays (500 r.).

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INCORPORATION OF THE PEPTIDE GLUTATHIONE INTO ISOLATED PROTEINS

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The ability of various isolated proteins to incorporate various amino acids under specific conditions has been shown in a number of reports [1-6].

The ability of isolated proteins to incorporate peptides, i. e., structural units of the protein molecule, larger than amino acids, has been found to be an essential problem in the investigation of the laws governing protein metabolism . Some thermodynamic and other indirect considerations were pointing toward the possibility of such a process.

Stahman [7] determined that crystalline proteins, such as chymotrypsin and serum albumin, will, under certain conditions, incorporate a polymer of glycine. In such proteins the amount of glycine increases while their nature and biological properties remain intact. In model experiments, Fruton [8-9] demonstrated the possibility of an interaction among polypeptides by the exchange of single peptide chains. He determined that this reaction consumed an insignificant amount of free energy.

Thus, the available data permits the assumption that not only amino acids but also higher structural units are the components from which protein synthesis orginates.

In the following work we investigated the ability of isolated proteins to incorporate peptides. We used a naturally occurring peptide composed of complex amino acids. The incorporation of the peptide glutathione which has a wide distribution and a great biological significance, by isolated proteins was studied. In the present article we report on the results obtained during the study of the incorporation of glutathione by proteins of plasma, hemolyzate, desoxypentose nucleohistone, pentose nucleoproteins and others under various conditions. The experiments were carries out with glutathione labeled with radioactive amino acids. Glutathione, containing glycine labeled with C^{14} in the carboxyl group and cysteine labeled with S^{35}, were prepared biologically.

Radioactive glutathione was prepared by injecting the labeled amino acid intraperitoneally four times at 45 minutes intervals into 2.5-3 kg rabbits. One hour after the last injection the animals were decapitated, their blood was collected and their liver was immediately isolated. Glutathione was extracted from the blood by defibrinating the latter, separating the erythrocytes by centrifugation, hemolyzing them, and precipitating the proteins by the addition of trichloroacetic acid to a final concentration of 10%. A mercaptan of glutathione was extracted from the protein-free filtrate according to the published method [10]. The same method was applied to extract the mercaptan of glutathione from the trichloroacetic acid filtrate of liver tissue. We succeeded in obtaining a mercaptan of glutathione giving 14 000-40 000 cpm/mg of tissue. The extraction of glutathione mercaptan from liver was 80-110 mg %.

In order to obtain free glutathione from mercaptan the latter was suspended in boiled distilled water and hydrogen sulfide was passed through this suspension. The black residue of metallic sulfides was discarded. The clear solution containing radioactive glutathione was used in the experiments.

Plasma was obtained from citrated rabbit blood, and the homolyzate, by hemolysis of repeatedly washed erythrocytes and subsequent separation from the stroma by centrifugation. Desoxypentose nucleohistone, pentose nucleoprotein, and elastin were prepared according to the directions contained in [2, 11].

The composition of the sample was: 25-30 mg of dry protein or 1 ml of plasma, depending on the hemolyzate; 3 ml of buffer solution according to Krebs, and labeled glutathione in the amount of

75 000-100 000 cpm per sample. In experiments with cysteine the radioactivity was of approximately the same order. The control samples were immediately precipitated with trichloroacetic acid of a final concentration of 10% and were kept at room temperature. The remaining samples were aerated by the passage of a mixture of O_2 and CO_2 (95 : 5%) and were incubated for 2 hours at 37° on a water bath.

At the end of the incubation period the proteins were precipitated by trichloroacetic acid of a final concentration of 10% and were repeatedly washed in a 5% solution of trichloroacetic acid, then three times with an alcohol-ether mixture (1 : 1) and finally twice with absolute ether. The samples with hemolyzate were washed with trichloroacetic acid, then 2 or 3 times with water, then were dissolved in alcohol and dried in thin layers. The radioactivity was determined with the aid of an ionization chamber on 5 mg samples of the obtained dry proteins, which were spread on aluminum discs.

Instruments of various sensitivities were used throughout the experiment for determination of radioactivity and appropriate remarks were made in the tabulation of the experimental material. During the radioactivity measurements of individual samples the square deviation did not exceed 10%.

TABLE 1

Incorporation of Gluthathione into Various Proteins

Expt. No.	Investigated proteins	Glutathione labeled with:	Temperature (°C)	Experimental	Control, precipitated by trichloroacetic acid
				cpm in 5 mg of protein	
1	Blood plasma	Glycine-C^{14}	37	14.0	2.5
	" "	"	37	11.0	1.3
2	" "	Cysteine-S^{35}	37	30.3	5.8
	" "	"	37	35.1	8.0
3	" "	"	37	188.0•	–
	" "	"	37	190.0•	–
4	" "	"	37	143.0•	45.0•
5	Fibrin	"	37	3.6	–
	"	"	37	4.6	–
6	"	"	37	30.0•	–
7	Hemolyzate	"	37	27.8	17.3
	"	"	37	33.3	24.6
8	"	"	37	162.0•	163.0•
				–	126.0•
	"	"	37	147.0•	147.0•
9	Pentose nucleoprotein	"	37	34.0	8.0
	"		37	36.0	7.0
	"	"	100	59.0	–
	"	"	100	60.9	–
10	Desoxypentose nucleohistone	Glycine-C^{14}	37	38.0	32.0
	"	"	37	37.0	32.0
	"	"	100	46.0	–
11	Elastin	"	37	5.2	4.0
	"	"	37	9.0	6.0

From the data in Table 1 it is apparent that proteins become radioactive after incubation with glutathione-C^{14} and -S^{35}. Elastin and fibrin exhibit less radioactivity than others. It is surprising that the control protein sample precipitated with trichloroacetic acid also displayed some radioactivity. The amount of radioactivity was not the same for various proteins. The radioactivity of plasma and pentose nucleoproteins

• The efficiency of the count was 4 to 5 times higher that that in other tests.

of control samples was significantly lower than in experimental samples, while the radioactivity of desoxynucleohistone and hemolyzate in experimental and control samples was almost the same.

In order to determine whether the radioactivity in the above mentioned proteins was due to the adsorption of glutathione (not removed during washings) or to the formation of disulfide or peptide bonds between glutathione and proteins, the radioactive proteins from experimental and control samples (Table 1) were reprecipitated and treated with ninhydrin and thioglycolic acid.

Protein precipitation was carries out as follows: the plasma or hemolyzate proteins were dissolved in 0.05 N NaOH, then precipitated by trichloroacetic acid, washed 3 times in a 5% solution of trichloroacetic acid, then washed twice in alcohol-ether (1 : 1) and twice in absolute ether. Desoxypentose nucleohistone, which is not completely soluble in an alkaline solution, was dialyzed for 24 hours in tap water, then precipitated with triochloroacetic acid and further washed as indicated above. The radioactivity of the treated dry protein was again measured. During reprecipitation of proteins in alkaline solution some breakage of disulfide bonds occurs and the proteins are freed of any adsorbed materials.

The ninhydrin treatment (varying the conditions of adsorption) was carried out according to the directions in [12]. The thioglycolic acid treatment (breaking the disulfide bonds between glutathione and proteins) was according to the directions in [13]. The results are given in Table 2.

TABLE 2

Radioactivity of Proteins after Reprecipitation and Treatment with Ninhydrin and Thioglycolic Acid

Expt. No.	Investigated proteins	Labeled compounds used	Before treatment	After treatment		
				with ninhydrin	reprecipitation	with thioglycolic acid
			cpm in 5 mg of protein			
1	Serum (expt.)	Glutathione-C^{14}	54	47	–	–
2	Blood plasma (expt.)	Glutathione labeled with cystein-S^{35}	188	131	–	–
	"	"	190	–	100	12
	"	"	143	–	–	12
3	Control	"	45	–	–	18
4	Hemolyzate (expt.)	Glutathione-C^{14}	455	420	–	–
5	Hemolyzate (expt.)	Cystein-S^{35}	162	157	90	91
	Control	"	126	114	90	60
	Hemolyzate (expt.)	"	147	–	–	55
	Control	"	147	–	–	56
6	Desoxypentose nucleohistone (expt.)	Glutathione labeled with glycine-C^{14}	152	–	64	–
	Control	"	128	–	44	–
7	Blood plasma (expt.)	Cystein-S^{35}	1966	–	–	90
	Control	"	336	–	–	28
8	Hemolyzate (expt.)	"	1026	–	–	833
	Control	"	341	–	–	133

As shown by the data of Table 2 the radioactivity of plasma proteins after the ninhydrin treatment as well as after reprecipitation remains at a significant level (> 50% of the initial radioactivity) which indicates that under the conditions of our experiments glutathione combines with plasma proteins mainly via the formation of chemical bonds.

However, the figures obtained upon thioglycolic acid treatment of plasma proteins indicate a retention of only 5-7% of the initial radioactivity. This indicates that the bond formed under the above described conditions between the plasma proteins and glutathione is basically due to the presence of a sulfhydryl group in the latter and quite insignificantly to other glutathione groups.

Table 2 shows that upon ninhydrin treatment of the hemolyzate proteins the radioactivity of the latter is changed little while reprecipitation leads to a significant decrease in radioactivity. However, even after the reprecipitation of hemolyzate proteins a significant radioactivity remains, amounting to over 50% of the initial activity. Upon thioglycolic acid treatment 30-40% of the radioactivity remains in the hemolyzate proteins. Therefore, during the interaction of glutathione with the hemolyzate proteins, the peptide combines with the proteins mainly through the formation of chemical bonds. However, contrary to the interaction of glutathione with plasma proteins, the peptide combines with the proteins of the hemolyzate not only through the formation of disulfide bonds, but also through the formation of a number of more stable bonds, possibly peptide linkages.

We were interested in comparing the addition of the amino acid cysteine to plasma and hemolyzate proteins with the addition of glutathione to the same proteins, inasmuch as cysteine like glutathione may be added to proteins with the formation of disulfide and apparently, peptide bonds. As seen from the Experiments 5 and 6 in Table 2 it combines quite strongly with plasma proteins, in the same way as glutathione, basically through the formation of disulfide bonds. However, the addition of cysteine to the hemolyzate proteins occurs through the formation of several stable bonds. The quantitative ratio of the latter bonds to the sulfhydryl bonds is approximately the same as in glutathione. Apparently the structure and composition of the protein determine the type of bond that will be formed in the process of addition of structural units.

The hemolyzate proteins in the control samples of Table 2, precipitated with trichloroacetic acid, also retained a significant amount of radioactivity after reprecipitation and treatment with ninhydrin and thioglycolic acid.

TABLE 3

Influence of the Addition of Non-Labeled Glycine and Cysteine on the Incorporation of Glutathione by Various Proteins

Exp. No.	Investigated proteins	Glutathione labeled with:	Non-labeled amino acid added, (mg)	Before thioglycolic acid treatment	After thioglycolic acid treatment
				cpm in 5 mg of protein	
1	Desoxypentose nucleohistone	Glycine-C	0	38	16.0*
			10, Glycine	37	13.5
2	Plasma	Cystein-S	0	148	12
			10, Cysteine	60	4
3	Serum	Glycine-C	0	63	–
			5, Cysteine	28	–
4	Serum	Glycine-C	0	63	–
			5, Glycine	58	–
5	Hemolyzate	Cystein-S	0	147	55
			10, Cysteine	59	18

The information obtained on the changes of radioactivity of hemolyzate proteins in the control samples indicates that the process of addition of glutathione to the hemolyzate proteins is not completely inhibited by trichloroacetic acid. Apparently, the addition of glutathione to desoxypentose nucleohistone is analogous to the latter mechanism. At the same time, the data in Table 1 shows that the addition of glutathione to plasma proteins as well as to pentose nucleoproteins is significantly inhibited by precipitation with trichloroacetic acid. This data shows that the interaction of glutathione with various proteins is not a simple one.

While investigating with isotopic tracers the mechanism involved in the addition of complex structural units (higher than amino acids) to proteins, the following problem arose: does not this radioactivity

* Desoxypentose nucleohistone was treated by reprecipitation, and not by thioglycolic acid.

result from the addition of a free labeled amino acid liberated during the degradation of a peptide chain? To resolve this question we performed several control experiments, although, in our experience with isolated proteins, no conditions favorable to the degradation of glutathione and the liberation of individual amino acids were present.

We used the method of isotope dilution in these control experiments [14]. The treatment with labeled glutathione was performed in the presence of non-labeled amino acids; glycine and cysteine. If the protein radioactivity in these experiments with labeled glutathione were due to the addition of a corresponding amino acid liberated from the latter, the addition of a non-labeled amino acid would have significantly decreased the radioactivity of these proteins. The obtained results are given in Table 3.

As seen from the data in Table 3, the amount of radioactivity in desoxypentose nucleohistone was the same whether or not non-labeled glycine was added.

This demonstrates that the radioactivity of desoxypentose nucleohistone results from peptide addition, and that under the conditions of this experiment no liberation of labeled glycine from glutathione could have taken place.

In those experiments where glutathione labeled with cysteine-S^{35} was used in the presence of non-labeled cysteine, the radioactivity of plasma proteins was approximately 40-33 % of that in samples not containing the extra non-labeled cysteine. This might give the impression that glutathione breaks down under the conditions of our experiment and that the radioactivity of plasma and hemolyzate proteins results from the addition of the amino acid cysteine formed during the degradation of glutathione (the label of cysteine might have been diluted by the addition of a non-labeled simple amino acid). However, further experiments on the effect of the addition of a non-labeled extra cysteine on the addition to proteins of a free labeled cysteine have shown that the protein radioactivity for instance in plasma, decreased under these conditions not by a factor of $2\frac{1}{2}$ - 3 but by a factor of 18-20. Moreover, a similar decrease in the radioactivity of the mentioned proteins during the addition of extra cysteine was observed in experiments where glutathione labeled with glycine-C^{14} was used.

It seems that the observed decrease in radioactivity of proteins in the samples containing extra non-labeled cysteine is due to a competitive action between cysteine and glutathione in the process of their addition at the same point of the protein molecule.

Therefore, under the conditions of our experiments, we may consider that it is the peptide and not a free amino acid which is incorporated in the presence of glutathione. However, the possibility is not excluded that not all of the tripeptide (glutathione) but only one of its component parts (dipeptide cysteinyl-glycine), is added.

Some investigations indicated that glutathione might act as a step in the synthesis of the protein molecule. It has been shown [15] that glutathione which is present during the action of triose phosphate dehydrase is strongly bound to the protein of this enzyme. The possibility of glutathione incorporation into proteins has also been demonstrated [12] by experiments on the incorporation of labeled glycine by various parts of the cell. These experiments established that the proteins became radioactive and that this radioactivity partly disappeared after destruction of the disulfide bonds. According to the authors this is the result of glycine-C^{14} incorporation into glutathione, which in turn is incorporated into the proteins, with the formation of disulfide bonds in the cell area under investigation. However, a direct proof of the presence of such a step-like process of protein synthesis has not yet been advanced.

The experimental evidence in our work indicates that the plasma proteins, hemolyzate proteins, desocypentose nucleohistone, pentose nucleoproteins and some others incorporate glutathione with the formation of disulfide and, apparently, peptide bonds. This allows us to consider that, under specific conditions, isolated proteins will incorporate peptides similarly to the incorporation of amino acids. In other words, protein synthesis is initiated from simple as well as from more complex structural units. It is characteristic that the process of glutathione incorporation by the above mentioned proteins may take place under various conditions. In a neutral solution it will add to all of the enumerated proteins. The addition of trichloroacetic acid (in a final concentration of 10%) will inhibit the addition of peptide only to some of them, for

instance to plasma proteins. On the other hand, this peptide will add to hemolyzate proteins in the presence of trichloroacetid acid almost with the same strength as in its absence. At the same time, the incorporation of glycine by these proteins will be inhibited by the addition of trichloroacetic acid [6]. The great variety of conditions under which the same reaction occurs is apparently due not only to the structure and composition of these proteins but also to the nature of the second component of the reaction. The influence of temperature on this process of peptide (glutathione) addition to proteins is also significant. While the incorporation of amino acids by desoxypentose nucleohistone and others is from $2\frac{1}{2}$-3 times as intensive at 100° as at 37°, the incorporation of glutathione by the same proteins is practically the same at both temperatures.

As a consequence the following problem arises: is it possible to consider the chemical processes of protein synthesis occuring in the presence of trichloroacetic acid or at 100° as enzymatic processes, or are these reactions occurring under the specified conditions independent of biological catalyzers? Since it has been previously shown that the activity of trypsin, chymotrypsin, pepsinogen and of the inhibitor of trypsin is not lost after boiling [16] and also that the activity of some enzymes is retained at 100° in an acid medium [17], there is a basis for the hypothesis that the incorporation of structural units into isolated proteins is catalyzed by an enzyme system retaining its activity under the specified conditions. However, we do not refute the fact that the rate of this reaction is increased in the presence of a biological catalyzer and that the same reaction may take place at great speed without any biological catalyzer, as has been established in the process of transamidation [18].

SUMMARY

Radioactive glutathione was isolated from the liver and erythrocytes of rabbits treated with glycine-C^{14} and cysteine-S^{35} In vitro experiments showed that glutathione is bound by isolated proteins of plasma hemolyzate, desoxypentose nucleohistone, pentose nucleoprotein, and elastin. The incorporation of the peptide (glutathione) by isolated proteins was shown to occur through the formation of disulfide and of more stable, apparently peptide, bonds. The relative quantities of these two kinds of bonds vary with the kind of protein. The process of peptide (glutathione)incorporation by some proteins is not inhibited by the action of a 10% solution of trichloroacetic acid.

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THE MECHANISM OF THE CARNOSINE EFFECT IN GLYCOLYTIC OXIDATION COUPLED WITH PHOSPHORYLATION

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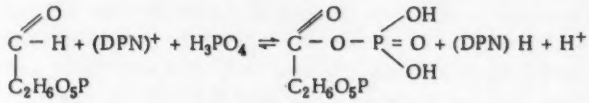
Due to a number of recent investigations, it has now been established that the nitrogen substances such as carnosine and anserine which may be extracted from muscle have a definite role in the functional activity of muscle tissue. Investigation of the function of these dipeptides in the biochemical processes leading to the accumulation of the energy used in muscular contraction is of great interest.

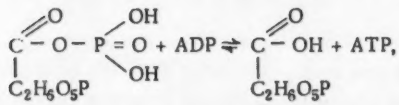
The investigations of Severin and his co-workers [1-4] established the accelerating effect of carnosine and anserine on glycolysis and aerobic phosphorylation. Homogenates or muscle extracts of various animals (rabbit, rat, pigeon, frog) were used as enzyme preparations for these experiments. A detailed investigation of the influence of these dipeptides on the anaerobic carbohydrate breakdown led to the discovery of the accelerative action of carnosine on the phosphoglucomutase reaction [1] on the transfer of the phosphate radical from adenosine triphosphoric acid (ATP) to creatine [1], and on the oxidation of phosphoglyceraldehyde [1,5,2]. The accelerating action of carnosine on the latter reaction is of the greatest interest as this reaction is one of the most important reactions of glycolysis since it is coupled with phosphorylation.

The action of carnosine on the oxidation of phosphoglyceraldehyde has been investigated by various authors. However, the mechanism of this reaction is still not understood. The present work is a further investigation of this problem.

An increase in the rate of any reaction may be obtained by acting on one of the factors inhibiting the rate of this reaction. Coupled glycolytic oxidative phosphorylation may be represented by the following scheme:



$$\begin{matrix} O \\ \| \\ C - H \\ | \\ C_2H_6O_5P \end{matrix} + (DPN)^+ + H_3PO_4 \rightleftharpoons \begin{matrix} O \\ \| \\ C - O - P = O \\ | \qquad\quad | \\ C_2H_6O_5P \qquad OH \\ \qquad\quad | \\ \qquad\quad OH \end{matrix} + (DPN)H + H^+ \qquad (1)$$



$$\begin{matrix} O \\ \| \\ C - O - P = O \\ | \qquad\quad | \\ C_2H_6O_5P \qquad OH \\ \qquad\quad | \\ \qquad\quad OH \end{matrix} + ADP \rightleftharpoons \begin{matrix} O \\ \| \\ C - OH \\ | \\ C_2H_6O_5P \end{matrix} + ATP, \qquad (2)$$

where DPN is a cozymase; (DPN)H is the reduced form of the cozymase; ADP is adenosine diphosphoric acid, and ATP is adenosine triphosphoric acid.

For a number of reasons (such as the values of the equilibrium constant of Reactions (1) and (2) and the strength of the bond between the enzyme catalyzing Reaction (2) and 1,3-diphosphoglyceric acid) the rate of the oxidation-reduction reaction depends directly on the conditions under which the transfer of the phosphate radical from 1,3-diphosphoglyceric acid to ADP is effected.

Thus, the presence of a phosphate acceptor is the factor determining the rate of the above reaction. However, there is another factor which influences the reduction rate of phosphoglyceraldehyde. It is the rate of oxidation of the reduced cozymase which is formed in this reaction. The oxidation of phosphoglyceraldehyde is effected by one of the three oxidation-reduction systems of glycolysis (I, II and III, Fig. 1). The hydrogen transfer is possibly

due to the presence of an oxidation-reduction potential difference between them. One system acts as a hydrogen acceptor, the other as a hydrogen donor.

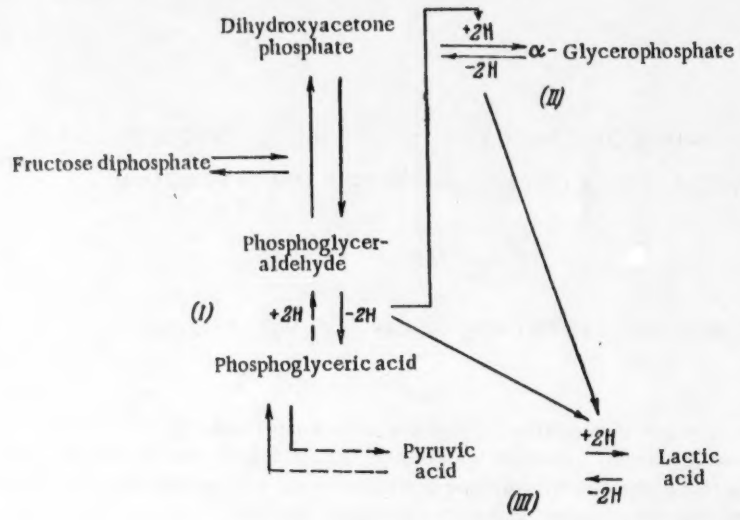


Fig 1. Cycle of oxidation-reduction reactions in glycolysis.

As a result of the oxidation of phosphoglyceraldehyde during glycolysis a reduced cozymase is formed which may in turn be oxidized during the reaction with dihydroxyacetone phosphate as well as during the reaction with pyruvic acid. Under normal physiological conditions the second path of oxidation takes place. Hydrogen is transferred to dihydroxyacetone phosphate only in case pyruvic acid is absent from the reaction media. This occurs during the initial or "induction" period of glycolysis. As pyruvic acid accumulates, it begins to react with the reduced cozymase. In the competition for hydrogen among the two possible acceptors - dihydroxyacetone phosphate and pyruvic acid - the latter is favored. For these reasons, the rate of the oxidation of phosphoglyceraldehyde during the induction period of glycolysis is several times slower than the oxidation rate in the presence of pyruvic acid.

In cases when the reaction media contains NaF, which exerts an inhibiting action on further transformations of phosphoglyceric acid and therefore on the formation of pyruvic acid, the conditions of the reaction become similar to those that are observed during the initial period of glycolysis. The rate of the oxidation-reduction depends on the presence of a phosphate acceptor as well as on the rate of oxidation of the reduced cozymase. The addition of NaF to the reaction media is convenient for the investigation of the carnosine effect since under these conditions it is possible to isolate its final product - phosphoglyceric acid. The addition of carnosine to samples containing NaF leads to an increased production of phosphoglyceric acid [2, 5]. While investigating the route of the carnosine effect in this reaction it is necessary to keep in mind both factors limiting its rate.

We first attempted to determine whether the action of carnosine on the oxidation-reduction reaction is coupled with its accelerating action on the oxidation of cozymase. If it were true, the acceleration of this reaction would be the result of a shift in the equilibrium of one of its oxidation-reduction reactions leading to the oxidation of the reduced cozymase (the reduction of dihydroxyacetone phosphate or the mechanism of lactic acid formation). In order to check the accuracy of this assumption we investigated the action of carnosine on the reaction between the reduced cozymase and pyruvic acid.

DPN was extracted from baker's yeast with an acidified mixture of alcohol and acetone, the coenzyme precipitated in the presence of an excess of alcohol and purified by adsorption on norite [6]. The preparation was 60% pure (by phosphorus and nitrogen). The hydrogenated form of cozymase was prepared by hydrosulfite treatment of its oxidized form according to the method of Euler [7] with several modifications.

The lactic dehydrogenase was extracted from heart muscle according to the method of Straub [8]. The enzyme was purified until a precipitate was obtained during the second precipitation with ammonium sulfate. For use in the experiment, part of the protein precipitate was dissolved in water and dialyzed against $\frac{1}{5}$ M phosphate

buffer (pH 7.2) for 5 hours in the cold. This solution was used as the enzyme preparation.

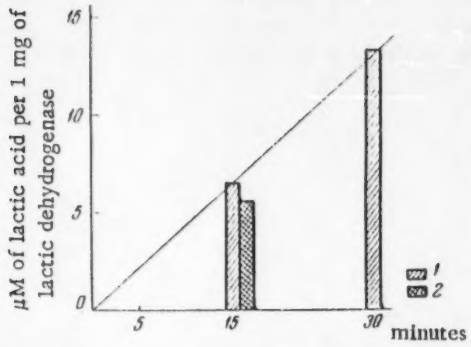


Fig. 2. Effect of carnosine on the reaction between (DPN)H and pyruvic acid.
1) without carnosine 2) with carnosine.

A 2.5 ml sample contained 3mg of sodium pyruvate, 5 mg of reduced cozymase and 0.07 M phosphate buffer (pH 8.2). A final 1% concentration of carnosine was used here as well as in all further experiments. The samples were incubated at 27-28° in the open air. The reaction was stopped by the addition of an equal volume of 5% trichloroacetic acid. Lactic acid was determined in the protein-free centrifugate according to the p-hydroxydiphenyl method [9].

No accelerating effect of carnosine in the oxidation of reduced cozymase with hydrogen transfer to pyruvic acid was observed during our experiments (Fig 2). However, these results did not exclude the possible action of carnosine in the reaction leading to the oxidation of the cozymase, i.e. the process of dihydroxyacetone phosphate regeneration. In order to verify this possibility, we duplicated the oxidation of phosphoglyceraldehyde independently from phosphorylation. It is known that the accelerating action of arsenic acid salts on glycolysis is due to the fact that the arsenate uncoupling the glycolytic oxidation-reduction from phosphorylation, renders its rate independent of the presence of mineral phosphate and phosphate acceptors. The arsenate, the action of which is analogous to that of the phosphate inReaction (1), makes the latter irreversible due to the fast hydrolytic dissociation of 1-arseno-3-phosphoglyceric acid. Reaction (2) does not occur in this case.

In the presence of NaF, the hydrogen from the reduced cozymase may be transferred only to dihydroxyacetone phosphate The rate of this reaction in the presence of arsenate depends only on one limiting factor, i.e., the oxidation rate of the reduced cozymase. If the action of carnosine were directed toward this process it should have been apparent in the experiments involving the addition of arsenate.

The enzyme systems used in the following experiments were obtained from dialyzed extracts of frog and rabbit muscle prepared in the following manner. Muscles of posterior extremities were cut into small pieces in the cold and homogenized in 1.5 times their volume of cooled distilled water for 30 minutes. The extract was centrifuged and dialyzed against tap water (6-7 hours) and against a 0.5% solution of KCl for 2 hours.

The reaction substrate, fructose diphosphate, was obtained through fermentation of beer yeast [9]. The preparation was 82% pure (by phosphorus and fructose).

The incubation was carried out at 29-30° in the open air. The reaction mixture consisted of equal volumes of an aqueous extract from rabbit or frog muscle ("enzyme extract") and a buffer solution of the following composition: 16 mg/ml of fructose diphosphate, 0.05M NaF, 0.4 mg/ml of cozymase, and buffer solutions of phosphate (0.12M), glycine (0.1M) or bicarbonate (0.2M) adjusted to pH 7.8. A final concentration of 4×10^{-4} M of Na_2HAsO_4 was added to the samples incubated with bicarbonate and glycine buffers. ATP(0.7 mg/ml of incubated mixture) and creatine (2.5 mg/ml of incubated mixture) were added to samples containing phosphate buffer. Fructose diphosphate and ATP were converted from barium salts to sodium salts before their use. After incubation, the proteins were precipitated by a constant volume of 5% trichloroacetic acid and the fructose diphosphate and phosphoglyceric acid were determined in the protein-free centrifugate [9].

It has been established by Severin and his co-workers that carnosine does not affect the aldolase reaction. Therefore, the large decrease in fructose diphosphate and the increase in phosphoglyceric acid observed in the presence of carnosine must be the result of the acceleration of the oxidation-reduction reaction. The results of our experiments presented in Fig. 3, indicate that the action of carnosine on the oxidation of phosphoglyceraldehyde cannot be detected in the absence of phosphate ions and phosphate acceptors. Therefore, the investigated reaction must be coupled with phosphorylation in order to observe the accelerating action of carnosine. Carnosine does not affect the oxidation of the reduced cozymase. Its action is apparently determined by the second factor inhibiting the rate of the coupled oxidative phosphorylation, i.e., by the transfer of the phosphate radical to the adenylic acid system.

Apparently the accelerating action of carnosine on the overall process (1) - (2)(p. 13, above) does not affect

its initial stage, i.e., the dehydrogenation of phosphoglyceraldehyde. This conclusion is supported by the evidence obtained during experiments on the reproduction of glycolytic oxidative phosphorylation in the reverse direction. If under such conditions carnosine would have retained its accelerating effect on the oxidation of phosphoglyceraldehyde, it would indicate that the dipeptide acted as a catalyst, i.e., regardless of the direction of the accelerating reaction. The carnosine effect could then be attributed to the acceleration of aldehyde dehydrogenation and the formation of reduced cozymase.

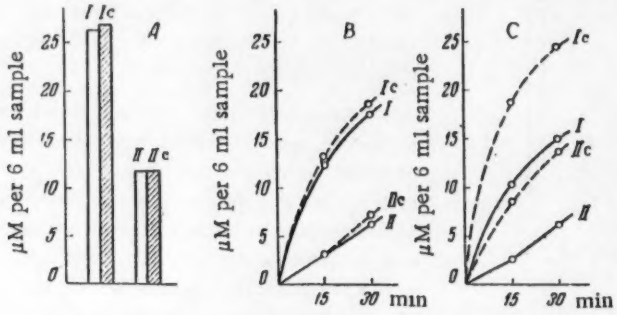


Fig. 3. Effect of carnosine on the decrease of fructose diphosphate and increase of phosphoglyceric acid under various conditions.
A) Bicarbonate buffer, enzyme extract prepared from frog muscle, incubated for 15 minutes; B) glycine buffer, enzyme extract of rabbit muscle, with twice its volume of water diluted; C) phosphate buffer, same extract as in B, but diluted with four times its volume of water.
I) Decrease of fructose diphosphate; II) increase of phosphoglyceric acid.
Ic and IIc) same, in the presence of carnosine.

In the following experiments we used enzyme extracts of frog muscle, dialyzed for 15 hours. The reaction media consisted of equal volumes of enzyme extract and of buffer solution of the following composition: 16 mg/ml of phosphoglyceric acid, 28 mg/ml of ATP, 0.05 M NaF, 0.3 M KCN, 0.06 M sodium lactate, 0.6 mg/ml of cozymase and 0.2 M bicarbonate buffer (pH 7.8).

ATP was added as a source of energy for the endothermic conversion of acid into aldehyde. The reduced cozymase indispensible for the reaction, was formed during the incubation from the added sodium lactate. KCN was used as a bonding agent for the reaction products (pyruvic acid and phosphoglyceraldehyde). Phosphoglyceric acid was obtained through fermentation of beer yeast [9]. There was no mineral phosphate present in the reaction. The amount of glyceric acid was found to be 105% and of phosphorus after mineralization 123% of the theoretically calculated values. ATP and phosphoglyceric acid were used in the form of sodium salts converted from barium salts. Sodium lactate was prepared from calcium lactate.

The incubation was carried out at 30° in the open air. The reaction was stopped by the addition of an equal volume of 5% trichloroacetic acid. Pyruvic acid (salicyl aldehyde method), phosphoglyceric acid and inorganic phosphate [9] were determined in the protein-free centrifugate.

The reaction was followed by observing the decrease of phosphoglyceric acid and the increase of pyruvic acid. These two processes occurred simultaneously (Fig. 4). We succeeded in following the process of inorganic phosphate splitting from ATP during the reaction (the values represented in Fig. 4 are corrected for the adenosinetriphosphatase splitting of ATP).

As seen from Fig. 4 carnosine appeared to inhibit this reaction. Apparently, this is the result of its accelerating action on the process of phosphoglyceraldehyde oxidation. The results of the latter series of experiments permit us to consider that the action of carnosine is not a catalytic action, and support the previously made conclusion that the accelerating effect of the dipeptide does not occur during the initial step of the reaction (the

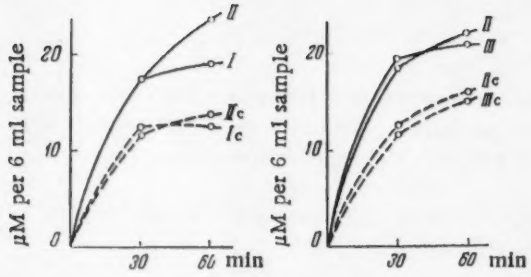


Fig. 4. Action of carnosine on the regeneration of phosphoglyceric acid.
I) Phosphoglyceric acid decrease; II) pyruvic acid increase; III) inorganic phosphate increase; Ic, IIc, IIIc) same, in the presence of carnosine.

process of aldehyde dehydrogenation). The carnosine effect is apparently related to an equilibrium shift resulting from the acceleration of one of the further stages of the reaction (organic bonding of the mineral phosphate or the transfer of the phosphate radical to ADP).

The assumption that the carnosine effect may be due to the fact that carnosine may act as an additional phosphate acceptor during ADP deficiency was investigated during the next series of experiments.

An extract of rabbit muscle, dialyzed for 5-7 hours, was used as the source of enzyme systems. The incubation was carried out at 21° in the open air. The reaction media consisted of equal volumes of enzyme extract from rabbit muscle and a buffer solution of the following composition: 16 mg/ml of fructose diphosphate, 0.05M NaF, 0.12 M phosphate buffer (pH 7.8). ATP and creatine were added to some of the samples in order to obtain ADP, the initial acceptor of the esterified phosphate. ATP was added in the amount of 0.7 mg per 1 ml of incubated mixture, and creatine in the amount of 2.5 mg per ml of incubated mixture.

As seen from Fig. 5 the reaction is quite slow in the absence of phosphate acceptor. It increases upon the addition of ATP and creatine. The addition of carnosine to samples not containing phosphate acceptors did not increase the rate of the reaction. The action of the dipeptide may be detected only in the presence of ATP and creatine. Thus, the results of the above experiments led us to the conclusion that the carnosine effect occurs at the stage during which the inorganic phosphate is esterified and ATP is formed.

Our next problem was to determine whether the action of carnosine is directed toward the acceleration of the transfer of the phosphate radical from 1,3-diphosphoglyceric acid to ADP or toward the acceleration of the esterification of inorganic phosphate and the formation of 1,3-diphosphoglyceric acid.

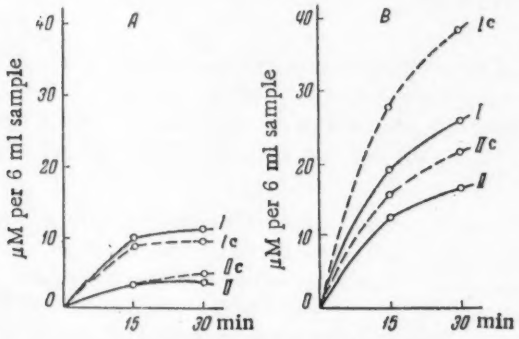


Fig. 5. The action of carnosine in the decrease of fructose diphosphate and the increase of phosphoglyceric acid, with and without the addition of ATP and creatine.
A) Incubation without creatine and ATP; B) incubation with addition of ATP and creatine; (the enzyme extract was dissolved in 6 times its volume of water). I) Fructose diphosphate decrease; II) phosphoglyceric acid increase; Ic, IIc) same, in the presence of carnosine.

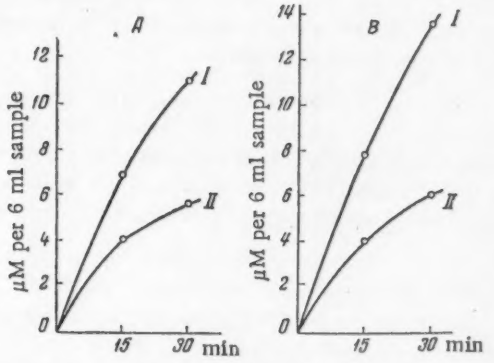


Fig. 6. Comparison of oxidation rates of phosphoglyceraldehyde in phosphate buffer (A) and glycine buffer containing arsenate (B). 10 ml of incubated solution (pH 7.3) contained 3.0 mg of aldolase, 10.5 mg of dehydrogenase and 3.5 mg of DPN.
I) Fructose diphosphate decrease; II) phosphoglyceric acid increase.

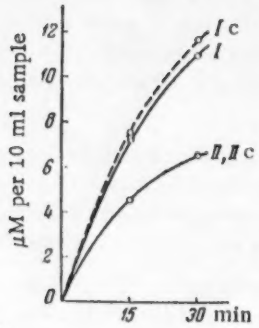


Fig. 7. Influence of carnosine on the oxidation reaction of phosphoglyceraldehyde in phosphate buffer. 10 ml of incubated solution (pH 7.3) contained 2.2 mg of aldolase, 11.5 mg of dehydrogenase, 3.0 mg of DPN.
I) Fructose diphosphate decrease; II) phosphoglyceric acid increase; Ic, IIc) same, in the presence of carnosine.

Significant advances have been made during the last years in the clarification of the mechanism of the action of phosphoglyceraldehyde dehydrogenase. The scheme advanced by Racker [10] and Segal and Boyer [11] divided the reaction catalyzed by this enzyme into two basic steps: 1) oxidation of the aldehyde with formation of thiol ester acyl-S-phosphoglyceraldehyde dehydrogenase; and 2) phosphorolysis of thiol ester with formation of 1,3-diphosphoglyceric acid and regeneration of the active group of the enzyme.

Our experiments with the addition of arsenate have demonstrated that carnosine does not affect the rate of the oxidation-reduction reaction leading to the formation of 1-arseno-3-phosphoglyceric acid, i.e., the absence of dipeptide takes no part in the formation of thiol ester and its arsenolytic dissociation. Furthermore we had to determine the relationship between the action of carnosine on the reaction occurring in phosphate buffer with acceleration of the phosphorolytic dissociation of thiol ester and the formation of 1,3-diphosphoglyceric acid. In order to observe the carnosine effect in the oxidation-reduction reaction leading to the formation of 1,3-diphosphoglyceric acid in the absence of phosphate transfer to ADP, it was necessary to exclude the possibility that the latter reaction would occur. Such conditions were found in the absence of ADP and of the enzyme transferring the phosphate radical from 1,3-diphosphoglyceric acid to ADP.

We performed the oxidation-reduction reaction catalyzed by a crystalline preparation of phosphoglyceraldehyde dehydrogenase. Under such conditions the reaction must stop at the stage of 1,3-diphosphoglyceric acid formation. If carnosine increases the rate of inorganic phosphate esterification, a greater amount of this ester must be formed, in its presence.

We isolated from rabbit muscle crystalline preparations of phosphoglyceraldehyde dehydrogenase and aldolase according to the method of Cori and others [12]. The recrystallized enzymes were kept in suspension in a solution of $(NH_4)_2SO_4$. Before the experiment, part of the crystalline suspension was separated by centrifugation (12-15000 r.p.m.) at 10° and dissolved in a buffer solution (0.1 M phosphate or 0.1 M glycine buffer, pH 8.0) cooled to 1-2° and containing cysteine in a 0.004 M concentration.

The reaction media consisted of equal volumes of a solution of phosphoglyceraldehyde dehydrogenase and a mixture of aldolase and fructose diphosphate solutions previously incubated for 10-15 minutes at 37° for the formation of some phosphoglyceraldehyde from fructose diphosphate to act as the substrate of the investigated reaction. The sodium salt fructose diphosphate, reconverted from its barium salt, was used in a final concentration of 0.005M. The reaction media also contained cozymase and Na_2HAsO_4 (in a final concentration of $4 \cdot 10^{-4}$ M) or either one of these compounds. The incubation was carried out at 20° in the open air. The reaction was stopped by the addition of a constant volume of 5% trichloroacetic acid. Fructose diphosphate and phosphoglyceric acid were determined in the protein-free centrifugate.

In the presence of crystalline dehydrogenase the oxidation of phosphoglyceraldehyde must culminate in the formation of 1,3-diphosphoglyceric acid. Since the reaction media did not contain either ADP or the enzyme transferring the phosphate radical to ADP, a hydrolytic dissociation of 1,3-diphosphoglyceric acid and the formation of 3-phosphoglyceric acid might have been expected. A hydrolytic dissociation of the arsenic derivative formed in the presence of arsenate also leads to the formation of 3-phosphoglyceric acid.

As shown in our experiments where the incubation was carried out simultaneously in phosphate buffer and in glycine buffer containing arsenate there was no significant difference in the rates of the reactions (Fig. 6).

Apparently the hydrolysis of 1-arseno-3-phosphoglyceric acid occurs only slightly faster than the analogous breakdown of 1,3-diphosphoglyceric acid. This would indicate that the hydrolysis of 1,3-diphosphoglyceric acid cannot determine the rate of phosphoglyceraldehyde dehydrogenation. Therefore, such experimental conditions may be indicated for the investigation of the carnosine effect in the esterification of inorganic phosphate.

However, it is necessary to create conditions under which the rate of the reaction would not be limited by the presence of a hydrogen acceptor. The added oxidized cozymase is able to interact with the reduced cozymase formed during the reaction and to displace it from the dehydrogenase. This explains the acceleration of the reaction observed in the samples with added cozymase. The acceleration is especially noticeable if the sample contains a catalytic amount of dehydrogenase with a small reserve of DPN attached to it. Such relationships were observed in the experiment tabulated in Table 1.

In experiments involving the addition of carnosine, the reaction media contained such amounts of dehydrogenase and cozymase that a further addition of DPN would not accelerate the reaction. Under such conditions, we expected that the addition of a factor able to accelerate the phosphorolysis of the thiol ester would increase the formation of phosphoglyceric acid. Fig. 7 shows that we observed no action of carnosine on the rate of the reaction occurring according to the above described scheme.

On the basis of these experiments we can assume that carnosine does not influence the esterification of inorganic phosphate. The action of this dipeptide on the coupled glycolytic oxidative phosphorylation apparently consists in acceleration of the phosphate transfer from 1,3-diphosphoglyceric acid to ADP. The fact that the carnosine effect depends on the presence of the phosphate acceptor supports this postulate. However, for a final elucidation of this problem, it will be necessary to reproduce the transfer of the phosphate radical from 1,3-phosphoglyceric acid to ADP in an isolated media and observe the action of carnosine on this reaction.

TABLE 1

Oxidation Rate of Phosphoglyceraldehyde in Phosphate Buffer, as a Function of the Amount of DPN Present. 10 ml of Incubated Solution (pH 7.8) Contained: 0.75 mg of Aldolase, 0.25 mg of Dehydrogenase; a) Without DPN; b) With 3.5 mg of DPN.

Time of incubation (minutes)	Fructose diphosphate decrease (μM)		Increase in the amount of phosphoglyceric acid (μM)	
	a	b	a	b
10	2.6	5.0	–	2.5
30	3.5	8.3	1.2	5.3

SUMMARY

An accelerating action of carnosine on the coupled glycolytic oxidative phosphorylation was observed in experiments with dialyzed extracts from rabbit and frog muscles. An inhibiting effect of carnosine was observed in the reverse reaction.

During uncoupling of the oxidative phosphorylation, occurring in the presence of arsenate, the accelerating action of carnosine disappears.

During experiments with a crystalline preparation of dehydrogenase it was impossible to detect any action of carnosine on that step of the oxidation of phosphoglyceraldehyde which leads to the formation of 1,3-diphosphoglyceric acid. It may be assumed that the accelerating action of carnosine takes place during the transfer of the phosphate radical to the adenylic acid system.

I wish to express my gratitude to Corresponding Member of the Acad. Sci. USSR, S.E. Severin, for suggesting the theme, assisting and supervising the course of this work.

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CHROMATOGRAPHIC ADSORPTION METHOD FOR THE QUANTITATIVE DETERMINATION AND SEPARATION OF ESTRINS IN THE URINE OF NON-PREGNANT WOMEN

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It is known that small amounts of estrogenic compounds up to a maximum of 200 μg per day, are being excreted in the urine of non-pregnant women. The urine also contains a number of various phenolic substances, pigments and chromatogens, the amount of which is influenced by a great many factors. The existing chemical methods of estrin* determination in urine are unable to take into consideration or eliminate this contamination by any means. As a result, a true quantitative analysis of hormones and their metabolites is impossible.

The presence of various phenolic and chromatogen substances in the urine and the imperfection of the present methods, make physiological studies of estrogen excretion very difficult. The increased use of hormones in clinical analysis has accelerated the development of more accurate methods of hormone analysis. At the present time the majority opinion is that the most thorough purification and separation of hormones is accomplished by the method of adsorption analysis [1].

The method of chromatographic adsorption was first applied for the extraction of steroid hormones by Duschinsky and Lederer [2]; they succeeded in extracting separately estrone and equilin from a mixture of hormones in the urine of a young mare. The adsorbing material was a hydrate of calcium oxide and the eluting agent was acetone. Heintzberger [3] applied chromatography for the quantitative determination of urine estrogens in order to achieve their further purification. The procedure is quite complicated and was not applied clinically. Stimmel published a number of investigations on the chromatography of urine estrogens [4]. His method consists of many steps. Separation and determination of three estrogens takes not less than 15-18 hours. Bitman and Sykes [5] developed a method allowing the separation and quantitative determination of estrone, estradiol and estriol in one column of 3 g of alkaline zeolite. The estrogen content was determined by a fluorimetric method.

We used in our experiments the data of Stimmel [4] on the adsorption characteristics of crystalline estrone, estradiol and estriol on a column of activated aluminum oxide. However, we introduced a number of substantial changes (the initial treatment of urine was radically changed): instead of 30 g of adsorbing material, we used only 3 g and changed the volume of the column as well as all the chromatographic conditions. The method of quantitative estrogen determination requires a smaller amount of reagents and less time, a factor which is necessary for its clinical application.

METHODS

A 250 ml sample of 24-hour urine, to which 15% by volume of concentrated hydrochloric acid was added, was hydrolyzed for 10 minutes. After cooling, both the acid and the neutral steroid were extracted from the hydrolyzate by ether which was washed twice with a saturated solution of sodium bicarbonate to avoid the formation of an emulsion. The acid phenolic steroids, or estrins, were extracted from the ether in two steps: they were first extracted with a 0.1 N solution of NaOH. The alkaline extracts were combined and acidified

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* According to the international nomenclature, estrin is a generic term for estrogen hormones.

with hydrochloric acid, and the estrogens extracted by ether. The ether was subsequently completely evaporated, the residue was dissolved in 3 ml of a 2% solution of methanol in benzene, and was passed through the column at the rate of 0.5 ml per minute. The 10/100 mm column containing 3 g of standard aluminum oxide of not less than 2nd degree activity was humidified beforehand with 3 ml of benzene. The adsorbing compound was then developed by 3 ml of benzene. The elution of hormones from the column was made by a solution of methanol in benzene; that of estrone by 15 ml of a 2% solution, that of estradiol by 10 ml of a 5% solution, and that of estriol by 10 ml of a 30% solution. The elutions were carried out gradually by 3 ml portions. Each subsequent portion of solvent was introduced into the column before the preceding portion was below the level of the aluminum oxide. The solvents containing estrone, estradiol and estriol were collected separately. The solvent was then distilled and the amount of estrogen determined according to the Cohen color reaction with half the volume of reagent on either a photocolorimeter or a photometer at 500 m μ.

Figure 1 summarizes the steps of estrogen determination by the method of chromatographic adsorption.

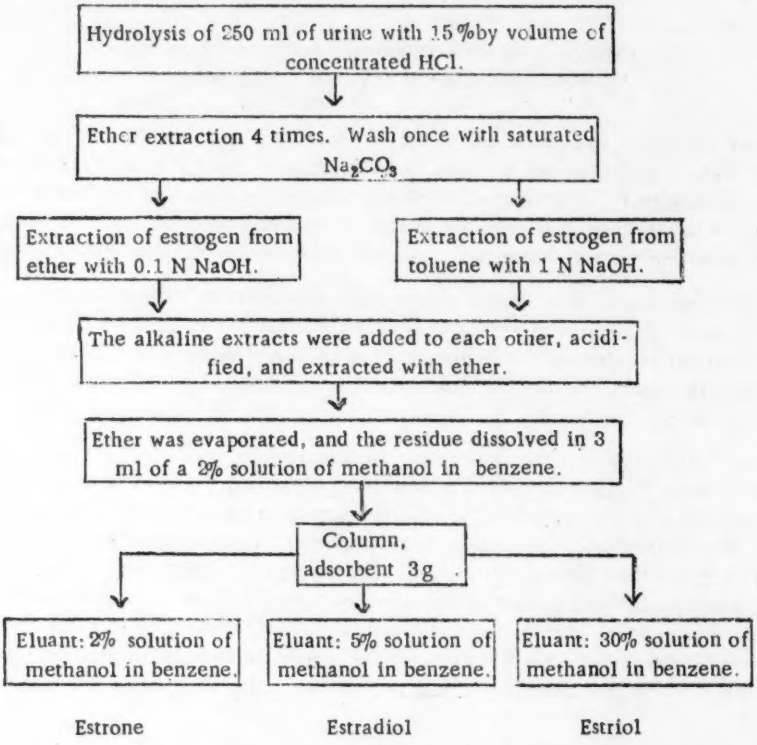
Hydrolysis of 250 ml of urine with 15% by volume of concentrated HCl.
Ether extraction 4 times. Wash once with saturated Na_2CO_3
Extraction of estrogen from ether with 0.1 N NaOH.
Extraction of estrogen from toluene with 1 N NaOH.
The alkaline extracts were added to each other, acidified, and extracted with ether.
Ether was evaporated, and the residue dissolved in 3 ml of a 2% solution of methanol in benzene.
Column, adsorbent 3g
Eluant: 2% solution of methanol in benzene.
Eluant: 5% solution of methanol in benzene.
Eluant: 30% solution of methanol in benzene.
Estrone
Estradiol
Estriol

Figure 1. Steps in estrogen determination by the chromatographic adsorption method.

Complete procedure of the quantitative determination and separation of estrogens takes not more than 5 hours (instead of 18). The price of reagents is also significantly reduced.

The method was experimentally verified with the following crystalline hormones produced at the Ukrainian Institute of Experimental Endocrinology: estrone, b. p. 254°, and estradiol, with b. p. 172°.

For determination of the degree of adsorption on 3 g of aluminum oxide, we performed 26 experiments with doses ranging from 20-100 μg of estrone and estradiol separately and simultaneously. It was demonstrated in all cases that the hormones are completely adsorbed.

It was necessary to determine the conditions of the hormone elution from the adsorbing material. Additional experiments with crystalline estrone and estradiol in which the eluting solution was collected and analyzed in 3 ml portions established: 1) that the hormones were not eluted by benzene; 2) that estrone is essentially not eluted by a 2% solution of methanol in benzene in 3 or 4 portions and is completely washed out after passage through the column of 15 ml of solution, while the estradiol begins to be displaced only after over 20 ml of solution (see Figure 2); 3) that estradiol is significantly better eluted by a 5% solution of methanol in benzene; it is

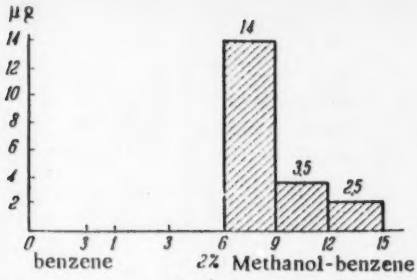

Figure 2. Chromatography of 0 μg of crystalline estrone. Elution by a 2% solution of methanol in benzene.

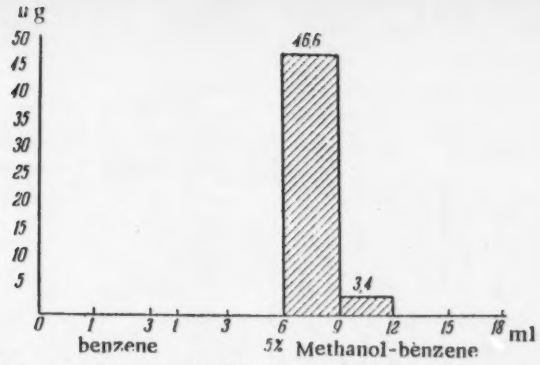

Figure 3. Chromatography of 50 μg of crystalline estradiol. Elution by a 5% solution of methanol in benzene.

almost completely washed off by 10 ml of this solution (see Figure 3). •

The results presented in Table 1 on the elution of crystalline estrone and estradiol show that under the conditions of our experiments we succeeded in eluting 91.5 - 100% of the hormones.

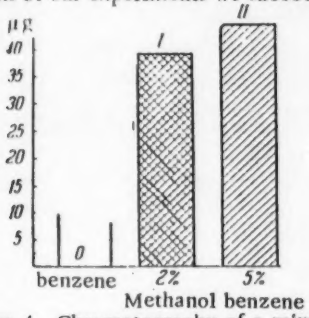

Figure 4. Chromatography of a mixture of crystalline estrone and estradiol (40 μg of each). Elution by solutions of methanol in benzene:
I) estrone; II) estradiol.

As is well known, the method of chromatographic analysis allows the separation of the complex hormone system into its individual components. The results of the separation of the two active forms of estrogen – estrone and estradiol, are presented in Figure 4.

The 2% eluant solution of methanol in benzene (6 portions of 3 ml, or 18 ml) were collected into one container and the 5% eluant solution (10 ml) into another. From the hormone mixture consisting of 40 μg of estrone and 40 μg of estradiol, 37 μg (92.5%) of estrone and 42 μg (105%) of estradiol were extracted.

It must be pointed out here that the absence of a complete separation of the active hormones (estrone from estradiol) should not be misinterpreted, since for clinical analysis the most important is not the separation of these forms one from each other, but the separation of the weak phenolic fraction of the most active (estrone plus estradiol) from the strong phenolic fraction of the least active (estriol).

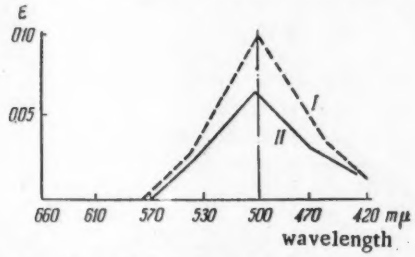

Figure 5. Absorption curves of crystalline hormones (estrone and estradiol) during the sulfuric acid color reaction:
I) estrone; II) estradiol.

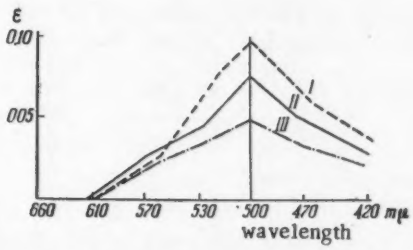

Figure 6. Absorption curves of hormones extracted from urine by the chromatographic absorption method, during the sulfuric acid color reaction:
I) estrone; II) estradiol; III) estriol.

• The estriol obtained in our laboratory from the urine of pregnant women and purified by the adsorption method, is not eluted by 15 ml of a 2% or 10 ml of a 5% solution of methanol in benzene. However it is displaced from the adsorbing material by a 30% solution of methanol in benzene.

TABLE 1

Elution of Crystalline Estrone and Estradiol.

No.	Hormone	Amount of hormone (μg)	Eluant: % of methanol in benzene	Amount of eluted hormone (μg)	Percentage of elution
1	Estrone	20	2	18.6	93
2	Estrone	20	2	20	100
3	Estrone	50	2	47.5	95
4	Estrone	100	2	91.5	91.5
5	Estradiol	20	5	20	100
6	Estradiol	50	5	50	100
7	Estradiol	100	5	95	95

TABLE 2

Recovery of Estrone and Estradiol Added to Urine

Expt. No.	Hormones	μg in urine	μg added	Total amt. of hormone (μg)	Recovered amount of added hormone		Total amount of hormone		
							were present,	were recovered	
					in μg	in %	in μg	in μg	in %
1	Estrone	16	50	66	71	108	126	119	94.5
	Estradiol	10	50	60	48	80			
2	Estrone	5	100	105	38	36			
	Estradiol	10	100	110	160	145	215	198	92.0
3	Estrone	5	50	55	40	73			
	Estradiol	6	50	56	70	125	111	110	99.9

TABLE 3

Comparative Results Obtained by the Method of Pincus and Pearlman and the Method of Chromatographic Adsorption with Column Fractionation (amounts in μg)

Expt. No.	Chromatographic adsorption method				Pincus and Pearlman method			
	estrone	estradiol	estriol	total	estrone	estradiol	estriol	total
1	5.0	15.5	8.3	28.8	3.6	11.1	9.2	23.9
2	7.2	6.7	4.2	18.1	4.3	8.9	3.3	16.5
3	5.7	11.1	4.2	21.0	4.3	10.0	4.2	18.5
4	7.2	7.8	4.2	19.2	–	8.9	3.4	12.3
5	10.0	14.4	3.4	27.8	–	7.8	2.5	10.3

However, further investigations are being carried out on the problem of a more complete separation of estrone from estradiol.

The basic proof of the accuracy of the quantitative method is a sufficient recovery of the added crystalline hormone. The analytical quantitative procedure is carried out on urine containing a known amount

of added crystalline hormone. The results of such experiments are presented in Table 2. The recovery of the added hormones reached 92 - 99.9%.

Since we did not dispose of crystalline estriol at the time of the experiment we were compelled to estimate the extraction of this compound on the basis of urinanalysis of non-pregnant women. Two equal volumes of such urine were analyzed, one by the method of Pincus and Pearlman [6] and the other by chromatographic analysis. The results of four experiments are given in Table 3.

The data of Table 3 show that the discrepancy in the amount of estriol determined by both methods does not exceed ± 0.9 g. The total amount of estrone and estradiol determined by the chromatographic method is somewhat higher than that obtained by the method of Pincus and Pearlman. This correspondingly increases the total amount of the three fractions by 10-20%.

The increase in total estrogen in urine analyzed by the chromatographic adsorption method is legitimately explained by the fact that the recovery of added hormones here reaches 90-95%, while according to published data and our data only 70-80% of the added hormones may be recovered by the Pincus and Pearlman method from the urine of non-pregnant women.

TABLE 4

Clinical Verification of the Method

No.	Diagnosis	Number of analyses	Estrogen amount in daily urine, in μg	Ratio of estrone + + estradiol to estriol
1	Regular and normal menstruations	8	61 – 221	1.4 – 1.9
2	" " " "	3	86 – 132	0.8 – 1.7
3	Inflammation of uterus without menstrual disturbances	7	19 – 142	0.8 – 1.8
4	" "	8	102 – 182	0.2 – 1.8
5	Inflammation of uterus with menstrual disturbances	8	57 – 118	1.6 – 4.2
6	Chronic inflammation of the uterus and liver	8	48 – 197	2.0 – 18.5
7	Ovary malfunction, metrorrhagia	4	59 – 125	2.2 – 4.0
8	Early sexual maturity, age: 2 years, 7 months; regular menstruations	1	89.3	4.8

An important criteria of the reliability of our method is the determination of the absorption spectra of the stained reaction products. The absorption maximum for the crystalline estrogen lies in the green spectral region (500 - 505 mμ) (see Figure 5).

The absorption curves of estrone, estradiol and estriol extracted from the urine of non-pregnant women by the method of chromatographic adsorption are presented in Figure 6. All three estrogens have absorption curves analogous to those of crystalline hormones.

Clinical Verification of the Method. The results of clinical verifications are presented in Table 4, the total amount of estrogen excreted in the urine and the ratio of the weak active fraction (estrone plus estradiol) to the strong least active (estriol) in women with normal menstruations and under various pathological conditions of the female organism.

From the above data it follows that in women with normal menstruation cycles the amount of hormone excreted in the urine does not exceed 220 μg; it occurs in the middle of the cycle and is apparently related to ovulation. The ratio of the active fraction (estrone + estriol) to estriol does not exceed 1.9. During ovary malfunction the ratio is significantly increased and reaches 4 and more in individual cases. However, the

amount of excreted estrogen remains within normal amounts (50-200 μg). Therefore, it seems to us that certain peculiarities of estrogen metabolism and excretion could be detected by the above described method.

SUMMARY

The use of an adsorption column for the purification and separation of various estrogen fractions significantly simplifies previous procedures, and at the same time reduces the amount of reagents used. The whole procedure of separation and quantitative determination of the estrogen fractions in the urine of non-pregnant women takes 5 hours instead of the 18 hours required by other methods.

The method allows determination separately, with an accuracy of 90-95% of the weak active fraction (estrone + estradiol) and the strong less active fraction (estriol). The absorption curves of the estrogens extracted from the urine of non-pregnant women correspond to the absorption curves of the crystalline hormones, which is an indication of their purity.

The results of clinical determinations of the total estrogen excreted and of the ratio of the two fractions permit the conclusion that the above method is well suited for the analysis of estrogen excretion.

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RESPIRATORY GAS EXCHANGE AND OXIDATIVE SYSTEMS OF TOMATOES

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Few biochemical investigations of tomato fruits have been published. Little data is available on the enzyme activity of tomatoes, on their respiratory quotients in the process of fruit growth, and on their ascorbic acid content [1,2]. However, improvement of the quality of food products and the decrease of losses during storage depend on knowledge of their biochemical activity. This statement was confirmed by the investigations of Sisakyan, Rubin, et al. (see for example [3-5]) and our work on the fermentation of citrus fruits [6].

The plant variety, its resistance, earliness of ripening, and fertility are directly related to the metabolic processes, in which respiration plays a dominant role. Therefore, we carried out some investigations on the character of the respiration and the oxidation-reduction processes in various tomato varieties which are widely used by the canning industry. At the same time we investigated the activity of metal-containing oxidizing enzymes such as peroxidase, polyphenoloxidase and ascorbinoxidase, and catalase and heavy-metal-free enzymes, such as flavoproteins. We also determined the content of free and bound ascorbic acid.

We investigated the new "Odessa 19" variety, and the "Mayak" variety, which is widely used in industry.

The tomatoes were analyzed at various developmental stages: in the bud stage, at the stage of small green fruits, at the stage of large green fruits, and then of pink and ripe fruits. The plants were all obtained from the same fields of the T.D. Lysenko Genetics Institute, Odessa in 1953 and 1954.

In addition to the investigation of the total rate of oxidation we were able to calculate the extent of the polyphenoloxidase and residual oxidation by investigating the action of diethyldithiocarbamate and potassium cyanide on oxidation.

The enzyme activities were determined by the following methods: that of polyphenoloxidase and peroxidase by the method of Mikhlin and Bronovitskaya [7] with the simultaneous investigation of the dynamics of the change in polyphenoloxidase activity in the Warburg manometer with pyrocatechin as the substrate; that of ascorbinoxidase by the amount of ascorbic acid destruction during the enzyme extraction; that of catalase by the iodometric method of Mikhlin and Pshenova [8]. The flavin enzymes were investigated in two ways: specific oxidative substrates for flavoproteins — glycocol, glucose, alanine, and asparagin — were introduced into the fruit tissues by the method of vacuum infiltration, and the oxygen absorption and the infiltrated water were determined in the Warburg apparatus. The inhibition of metal-containing systems was simultaneously effected by potassium cyanide. Ascorbic acid was determined by titration with dichlorophenolindophenol.

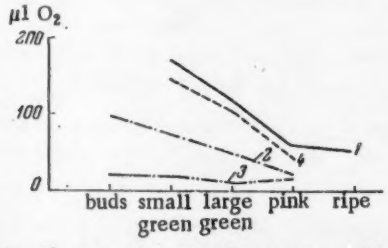

Fig. 1. Changes of respiration pattern with growth of "Mayak" tomatoes. 1) Total respiration; 2) polyphenoloxidase respiration; 3) residual respiration; 4) respiration catalyzed by metalloproteins.

The results of our investigation of the respiration (on the basis of 4 or 5 repetitions) are presented in Figs. 1 and 2; it follows from these experiments that the total oxygen consumption decreases as the tomato fruit ripens. The rate of total respiration in the stage of small green fruits is almost twice as large in the "Odessa 19" variety as in the "Mayak" variety; this rate is approximately equal in ripe fruits of both varieties. At this stage the "Mayak" fruits somewhat increase their rate of respiration. All the stages investigated are characterized by polyphenoloxidase and flavoprotein but their rates vary during the growth process.

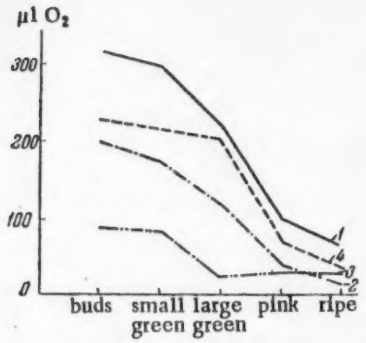

Fig. 2. Changes of respiration pattern with growth of "Odessa 19" tomatoes. 1) Total respiration; 2) polyphenoloxidase respiration; 3) residual respiration; 4) respiration catalyzed by metalloproteins.

Of basic importance in the bud is respiration catalyzed by the metal containing enzymes. In "Odessa 19" fruits, the flavoprotein rate of oxidation is more than twice that of the "Mayak" fruits. At this stage the polyphenoloxidase oxidation rate is more than twice that of the oxidation catalyzed by the metal-containing enzymes, reaching 63% for the "Odessa 19" variety.

No significant changes in the activity of various oxidative systems occur during the stage of small and large green fruits in comparison with the preceding stages. In the "Mayak" variety the transition to the pink fruit stage is accompanied by a noticeable drop in the rate of oxidation catalyzed by the metal-containing enzymes, while no such significant changes are observed in the "Odessa 19" variety. Furthermore, the residual oxidation increases in the former variety and remains almost constant in the latter. During this stage the polyphenoloxidase oxidation continues to decrease in both varieties. As the fully ripe stage approaches in the "Odessa 19" variety, the oxidation catalyzed by metal-containing enzymes decreases. Judging by the increase of oxygen absorption in the presence of amino acids, the flavoprotein activity during this period increases; however, the first type of oxidation remains predominant.

The data on flavin enzyme activity are presented in Table 1. The oxygen absorption in the presence of KCN indicates their low activity during the bud stage, while their activity significantly increases toward the stage of full ripening.

Experiments with addition of amino acid give analogous results, i.e., as the fruit ripens the oxygen absorption, upon addition of substrate, begins to increase, reaching a significant rate at the fully ripe stage.

From the work of Rubin [5] who demonstrated that the flavin enzymes are more resistant to adverse conditions than the metal-containing enzymes, one can conclude that the "Odessa 19" variety is more resistant to low temperatures than the "Mayak" variety. Thus, the former variety has a substantial advantage over the latter, since it bears fruit just before frost sets in.

In certain cases, our data contradicted the general conclusions. For instance, in one of the experiments we observed an increase in oxygen absorption, while KCN inhibited the gas exchange catalyzed by the metal-containing enzymes. In a number of experiments we observed a decrease in oxygen absorption during the infiltration of glucose, alanine and others. In one experiment we observed a 50% increase in oxygen absorption in the presence of alanine, while the oxygen absorption remained small in presence of KCN.

Rubin [4], Bereznegovskaya [9] and others encountered the same phenomena during their investigations. Apparently their explanations of these phenomena can be extended to our experiments. The increase in oxygen absorption in one of the above-described experiments could be explained by the extent to which the flavin enzymes, capable, in certain cases, of being activated by KCN, are active in oxidation-reduction processes occurring in tomato fruits.

The enzyme composition is identical in both varieties and remains constant during the whole process of ontogenesis, except for peroxidase, the activity of which approaches zero in the stage of bud formation. The maximum activity of this enzyme in both varieties is at the stage of pink fruits. Subsequently its activity sharply decreases in the "Odessa 19" variety, while it continues to increase in the "Mayak" variety.

The activity of the remaining enzymes, especially of flavoproteins, during this period is higher in the "Odessa 19" fruits. Apparently the peroxidase system is replaced by other oxidative systems in the fruits of the latter variety.

In the bud stage the polyphenoloxidase activity is high in both varieties. However, it is 1.5 times as high in the "Odessa 19" variety as in the "Mayak" variety, which is in conformity with the results of the polyphenoloxidase respiration investigations. At the ripe fruit stage the activity of this enzyme is almost identical in both

varieties. The catalase activity decreases in both varieties during the growth process and reaches the same level at the stage of pink fruits after which a four-fold increase is observed toward the period of full ripening. According to Gustafson[10] there is a direct relationship between the catalase activity and the energy of respiration. The ascorbinoxidase activity remains almost identical in both varieties during thewhole process of ontogenesis.

TABLE 1

Flavin Enzyme Activity in Tomato Buds and Fruits During Their Growth Process. (The figures for the O_2 absorption changes due to the addition of substrate are percentages of the figures obtained from water-containing samples)

Substrate	"Mayak"					"Odessa 19"				
	buds	stages of fruit development				buds	stages of fruit development			
		small green	large green	pink	ripe		small green	large green	pink	ripe
Glucose	100	–	103	–	122	103	–	–	125	318
Glycocoll	–	100	101	–	–	114	–	–	125	–
Alanine	102	111	–	–	137	–	100	112	–	381
Asparagin	–	110	108	–	–	–	112	–	100	318

The published data on the characteristics of plant enzyme systems are in some cases similar to ours, for instance the work of Pontovich [11] on the activity of biocatalyzers in krym-saghyz cuttings. The data of Sisakyan and Rubin [12] on the polyphenoloxidase and peroxidase activities differ from our results. The investigations of one of the members of our group on the activity of oxidizing enzymes in the ripening process of grapes show that the catalase and polyphenoloxidase activity are similar to the same enzymes in tomato plants. The shift of oxidative systems in the process of plant growth, demonstrated by Sisakyan and Rubin [12], is confirmed for tomato fruits.

TABLE 2

Ascorbic Acid Content in Tomato Buds and Fruits During Their Ripening Process.

Experimental subject	Ascorbic acid, in mg %		Respiratory quotient in μl O_2 per 100 mg
	Free	Bound	
"Mayak" buds	18.1	5.8	–
"Odessa 19" buds	17.4	0.9	317
"Mayak" small green fruits	–	–	170
"Odessa 19" small green fruits	18.1	2.4	300
"Mayak" large green fruits	29.7	3.8	120
"Odessa 19" large green fruits	27.2	–	232
"Mayak" pink fruits	–	1.03	44
"Odessa 19" pink fruits	33.49	–	95
" Mayak" ripe fruits	28.5	0.85	50
" Odessa 19" ripe fruits	29.96	4.4	60

On comparing the changes of activity of the various oxidases with curves of various types of oxidation one can see that the highest ascorbinoxidase and polyphenoloxidase activities occur during the time of greatest synthetic activity in the fruit tissues. Later on, during the vegetative period, the catalase activity together with the latter processes, characterize the respiration.

Our results indicate the absence of any relationship between the intensity of respiration and the peroxidase activity. This enzyme, according to Mikhlin and Bronovitskaya [7] evidently reacts with hydrogen peroxide which is formed by the flavoprotein enzymes, and forms with them a coupled oxidative system.

The curve of polyphenoloxidase activity resembles the respiration curve: polyphenoloxidaserespiration accounts at all stages for more than 50% of the total respiration in which the metal-containing enzymes also participate. On this basis we can assume that the polyphenoloxidase type of respiration is predominant in tomatoes.

According to some investigators the same respiratory system is found in potato tubers [13-14].

It has been shown [15] that the rate of respiration in plant tissues is related to the content of ascorbic acid which, according to a number of authors [16], is the catalyst of oxidation-reduction processes.

Table 2 shows the changes of the ascorbic acid content during the process of tomato ripening. There is a noticeable increase during the initial period of fruit development. A further insignificant accumulation occurs in the tissues toward the time of full ripening. At the same time a decrease in the rate of respiratory gas exchange in both varieties is observed. A similar situation in R. cinnamomea has been reported in the literature [15]. A possible explanation is that a small amount of ascorbic acid is active in the oxidation-reduction processes, while the remaining ascorbic acid combines various compounds to form a complex and becomes an inert reserve substance.

Investigations of the bound form of ascorbic acid in the same tomato varieties have shown that its changes are opposite to that of the unbound form. In "Odessa 19" fruits it accumulates toward the stage of complete ripening. On the basis of the hypothesis of some authors that the bound form of ascorbic acid remains intact due to its resistance to oxidation, we can conclude that the "Odessa 19" variety has a greater ability to retain ascorbic acid than the "Mayak" variety. The above biochemical investigations on tomato fruits may be applied in determining the optimal regime for the storage of tomato products.

SUMMARY

The rate of respiratory gas exchange decreases as the tomato fruit ripens. At the time of ripening the oxidation catalyzed by metal-containing enzymes decreases while the residual oxidation catalyzed by flavin enzymes increases. The share of polyphenoloxidase activity in the oxygen absorption by all oxidative systems in tomato fruits is quite significant (over 50% of the total respiration). The activity of this enzyme is most closely related to the process of respiratory gas exchange, as shown by the enzyme's activity curve.

Different tomato varieties have different biochemical properties. At the stage of full ripening there is a high activity of flavoprotein enzymes in the "Odessa 19" variety. This is an important property, which may permit extending its cultivation season. This variety is apparently better able to retain ascorbic acid than is the "Mayak" variety.

In conclusion we would like to express our gratitude to the workers of the Vegetable Section of the T.D. Lysenko Genetics Institute of Odessa for the assistance given during the course of the present work.

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HIGHER FATTY ACIDS OF THE DIPHTHERIA BACILLUS

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The Russian authors Dzerzhgovsky and Rekovsky [1] pioneered in the investigation of diphtheria bacilli lipids. Later on Chargaff [2] isolated tetradecanoic acid, palmitoleic acid, palmitic acid, diphtheric acid $C_{35}H_{68}O_2$ and corynine $C_{50}H_{100}O_4$ acid from the lipids of the diphtheria bacillus. Gubarev and Vakulenko then demonstrated the presence of corynic acid, after which Gubarev [4,5] discovered a group of hydroxy diphtheric acids which proved to be effective anti-leprosy agents [5,6]. Asano and Takahashi [7,8] discovered myristic acid, behenic acid, lignoceric acid, heneicosanoic acid and octacosanoic acid and confirmed the presence of diphtheric acid, in the lipids of diphtheria bacilli. Gubarev and his co-workers [9] discovered the α- and β- groups of corynic and hydroxycorynic acids and also demonstrated the presence of acids with the following formulas: $C_{19}H_{38}O_2$, $C_{21}H_{42}O_2$, $C_{21}H_{40}O_2$, $C_{22}H_{42}O_2$, $C_{25}H_{48}O_2$, $C_{26}H_{52}O_2$, $C_{35}H_{66}O_2$, $C_{38}H_{76}O_2$ and $C_{23}H_{44}O_2$ [10].

Lederer and Pudles [11,12] discovered corynomycolic ($C_{32}H_{64}O_3$) and corynomycolenoic ($C_{32}H_{62}O_3$) hydroxy acids in the lipids of diphtheria bacilli. These authors have lately isolated the ketone: palmitone $C_{31}H_{62}O$ and cis- Δ^7-palmitenone ($C_{31}H_{60}O$) [13].

Kanchukh [14] isolated arachidic acid and also acids with the following formulas: $C_{31}H_{60}O_3$, $C_{33}H_{64}O_3$, $C_{33}H_{66}O_3$, $C_{36}H_{72}O_3$ and $C_{50}H_{100}O_2$.

It must be pointed out that a number of the above formulas should not be taken for granted since in some cases the authors were isolating mixtures of fatty acids or mixtures of fatty acids with neutral substances. Several newly-developed methods such as precise fractional vacuum distillation [15,16], low-temperature crystallization [17] and chromatography [18,19] permit a much more accurate investigation of the bacterial lipids.

Using the above-cited methods, we investigated the higher fatty acids of the lipids of diphtheria bacilli (PW8 strain), obtained from various microbiological institutes.

Bacteria from a 10-12 day culture on Martin's beef-peptone broth were filtered, washed several times with distilled water and dried at 100°. The bacterial mass was received in this state at our laboratory where it was further crushed into a fine powder.

3.0 kg of bacteria was divided into 3 portions, each of which was extracted three times with benzene by infusing at room temperature for 72 hours. 60 g of lipids in the form of a brown waxy substance was obtained from the combined extracts after distillation of the benzene. These lipids were saponified by boiling for 15 minutes with 0.7 liter of 1N KOH solution in ethyl alcohol under reflux. The carbohydrate precipitate which formed during saponification was filtered off. The obtained alcohol solution of soaps and unsaponified substances was evaporated down to $\frac{1}{3}$ of its initial volume, diluted with 5 times its volume of water, and then extracted in a separatory funnel with ethyl ether for the extraction of unsaponified substances. Neither these ether-soluble substances nor the carbohydrate precipitate were investigated further.

20.6 g of free higher fatty acids was obtained from the aqueous solution in the usual way; a brown substance with a melting point of 50-53°, a neutralization number of 138.5 and an iodine number of 30.4.

19.2 g of higher fatty acids was converted into methyl esters and then subjected to precise vacuum-

fractionation in a column [16]. On distilling 18.15 g of the methyl esters of the higher fatty acids, we obtained 4 fractions of distillate of a total weight of 9.24 g (wetting of the packing at the rate of 30-50 drops per minute, reflux number 15: 1-5:1, pressure at head of column 0.5 mm Hg) (see Table 1).

The distillation of the mixture was carried out for 5 hours and 55 minutes at a temperature of 250°. The distillation residue was a dark brown semi-liquid substance, weighing 7.50 g.

TABLE 1

Properties of the Fractions Obtained Upon Distillation of the Methyl Esters of Higher Fatty Acids from the Lipids of Diphtheria Bacilli

Fraction	Temperature of vapors in head of column(°C)	Weight (g)	Appearance
I	114.5-115	2.83	Clear, light yellow oil
II	115-118	3.10	Ditto
III	118-125	1.83	Clear, light green oil
IV	142-147	1.48	Clear, green oil

Investigations of the Higher Fatty Acids Distillate

The obtained distillate fractions were further separated by the method of low-temperature crystallization of a 3-5% acetone solution of the methyl esters and then of the free fatty acids.

The isolated higher fatty acid methylester fractions were then saponified by heating for 1 hour with a 1 N solution of KOH in ethyl alcohol (2 ml of basic solution per 100 mg of saponified substance). The free higher fatty acids were separated in the usual way from the products of saponification. The obtained fractions were further investigated by our method of paper chromatography [19]. The melting point determinations were carried out in capillaries. The temperature when the miniscus formed over the column of melting substance was taken as the initial melting point, and that at the moment of the complete clearing of the substance as the final melting point. Iodine numbers were determined according to the method of Ganus as modified by Chargaff [20] for microdetermination.

Analysis of Fraction I

The free acids were obtained by low-temperature crystallization of the higher fatty acid methyl esters of Fraction I, and the saponification of the resulting fine fractions. Their properties are presented in Tables 2 and 3.

TABLE 2

Properties of the Methyl Esters of Higher Fatty Acids Obtained by Low-Temperature Crystallization of Fraction I

Fraction	Crystallization temperature of the residue (°C)	Weight (g)	Color	Consistency at +20°
I-a	−3 −5	0.320	Brownish-yellow	Hard
I-b	−10 −13	1.029	Light yellow	"
I-c	−20 −22	0.199	Light yellow	"
I-d	residue	1.123	Yellow	Liquid

The higher fatty acids of Fraction I-b and I-d were further separated by low-temperature crystallization from a 5% solution in acetone (Table 4).

The physical and chemical properties of Fraction I-a suggest that this fraction could be palmitic acid containing some palmitoleic acid. Two spots, characteristic of palmitic acid (R_F 0.52; 0.50) and palmitoleic acid (R_F 0.83; 0.81) were detected by paper chromatography (90% methyl alcohol development). That Fraction I-b-I was pure palmitic acid was also demonstrated by paper chromatography. Fraction I-c corresponded in properties to palmitic acid containing some palmitoleic acid. Its higher neutralization number indicated

TABLE 3

Properties of Free Fatty Acids Obtained from the Table 2 Fractions

Fraction	Weight (g)	Color	Consistency at +20°	M.p. (°C)	Neutralization No.	Iodine No.	Refractive index, n_D^{70}
I-a	0.250	Light yellow	Hard	59.5-60	218.3	19.3	1.4325
I-b	0.754	"	"	—	221.1	9.1	—
I-c	0.135	"	"	60-61	226.0	10.7	1.4318
I-d	0.891	Reddish brown	Soft	—	220.3	71.3	—

TABLE 4

Properties of Purified Preparations of Palmitic and Palmitoleic Acids.

Fraction	Crystallization temperature (°C)	Weight (g)	Color	M.p. (°C)	Neutralization No.	Iodine No.	Refractive index, n_D^{70}
I-b-I	−5 −10	0.681	White	62.7	219.1	0	1.4304
I-b-II	residue	0.048	Lt. brown	41.5-47	—	—	—
I-d-I	−12 −15	0.127	Lt. yellow	56-57	219.3	—	—
I-d-II	−22 −26	0.032	"	49-51	—	—	—
I-d-III	residue	0.525	Red	+2 +4	219.5	86.4	1.4569

that it might contain some fatty acids of low molecular weight. However, paper chromatography (90% methyl alcohol development) revealed only two spots characteristic of palmitic (R_F 0.45; 0.39) and palmitoleic (R_F 0.83; 0.81) acids. Fraction I-d-III was almost pure palmitoleic acid. Paper chromatography (75% methyl alcohol development) revealed only one spot characteristic of this acid (R_F 0.65). The residues of Fractions I-d-I and I-d-II were mixtures of palmitic and palmitoleic acids which was verified by paper chromatography.

Therefore, Fraction I was a mixture of palmitic and palmitoleic acids, the methyl esters of which distilled at 114.5-115° at a pressure of 0.5 mm Hg. On the basis of these values it is easy to calculate the vapor temperatures of the methyl esters of the fatty acids with 18 and 20 carbon atoms. By a special procedure of methyl palmitate and methyl stearate distillation at a pressure of 0.5 mm Hg it was determined that there is a difference of only 12.5-13° in their boiling points. Therefore, we expected the methyl esters of the higher fatty acids with 18 carbon atoms per molecule to distill at a temperature of 125-130°, and the esters of acids with 20 carbon atoms to distill at 140-145°. These calculations were found to be in accordance with the results of the analysis of further fractions. The absence of distillate in the 125-142° temperature range indicates the absence of significant amounts of higher fatty acids with 18 carbon atoms in the investigated mixture.

Investigation of Fraction II

The free acids were obtained by low-temperature crystallization of the methyl esters of the Fraction II higher fatty acids and the saponification of the resulting fine fractions. Their properties are presented in Table 5. The acids of Fraction II-a were subjected to further low-temperature crystallization from a 5% solution in acetone (Fraction II-a-I, coming down at −1 −5°, and the II-a-II fraction, which is the residue).

TABLE 5

Properties of the Free Higher Fatty Acids of Fraction II

Crystallization temp. of the methyl esters residue, (°C)	Fraction	Weight (g)	Color	M.p. (°C)	Neutralization No.	Iodine No.	Refractive index, n_D^{70}
−1 −6	II-a	1.062	Lt. yellow	—	219.0	9.15	—
	II-a-I	0.871	White	62.1	219.9	0	1.4309
	II-a-II	0.177	Lt. brown	54-59	214.3	35.8	1.4352
−10 −11	II-b	0.248	Lt. yellow	60	220.8	14.8	—
residue	II-c	0.127	Lt. yellow	50-51	220.5	54.6	1.4352

Fraction II-a-I was found to be pure palmitic acid. Fraction II-a-II was a mixture of palmitic and palmitoleic acids, which was confirmed by paper chromatography. Fraction II-b was palmitic acid containing a small amount of palmitoleic acid. Fraction II-c was a mixture of palmitic and palmitoleic acid with an unknown acid. Paper chromatography of Fraction II-c (75% methyl alcohol development) revealed 3 spots: one, characteristic of palmitic acid (R_F 0.06; 0.09); the second had an R_F of 0.45, 0.47; and the third, R_F 0.74, 0.78. Upon development of the chromatogram with 90% methyl alcohol, in addition to the spot corresponding to palmitic acid (R_F 0.43; 0.50), two other spots were detected: R_F 0.60, 0.65; and 0.81, 0.83. No detailed analysis of the components of this mixture was attempted due to the small quantity of the substance remaining.

Analysis of Fraction III

The methyl esters of the higher fatty acids of this fraction were separated by low-temperature crystallization from a 3.7% solution in acetone and converted into free acids by the usual methods (see Tables 6 and 7).

TABLE 6

Properties of the Methyl Esters of Higher Fatty Acids Obtained Upon Low-Temperature Crystallization of Fraction III

Fraction	Crystallization temperature of the residue (°C)	Weight (g)	Color	Consistency at +20°C
III-a	−5 −10	0.924	Light yellow	Hard
III-b	−15 −17	0.210	"	Hard
III-c	Residue	0.687	"	Liquid

TABLE 7

Properties of Free Fatty Acids in Fraction III

Fraction	Weight (g)	Color	Consistency at +20°C	M.p. (°C)	Neutralization No.	Iodine No.
III-a	0.804	Lt. yellow	Hard	—	216.3	5.5
III-b	0.172	"	"	60-60.5	216.3	11.0
III-c	0.586	"	"	—	204.8	65.5

The higher fatty acids of Fractions III-a and III-c underwent an additional low-temperature crystallization; Fraction III-a from a 5% acetone solution and Fraction III-c from a 3.3% acetone solution. Fractions III-c-III and III-c-IV were obtained by a subsequent low-temperature crystallization from a 5.3% acetone solution of Fraction III-c-II (Table 8).

TABLE 8

Low Temperature Crystallization of the Higher Fatty Acids of Fraction III

Fraction	Crystallization temperature of the residue (°C)	Weight (g)	Color	M.p. (°C)	Neutralization No.	Iodine No.	Refractive index, n_D^{70}
III-a-I	−16 −20	0.148	Lt. yellow	62.4	212.0	—	1.4310
III-a-II	Residue	0.023	"	56-57	—	—	—
III-c-I	−25 −29	0.080	"	57.5-58	191.4	20.3	1.4350
III-c-II	Residue	0.321	"	25-36	—	—	—
III-c-III	−32 −33	0.050	White	50.5-53	193.4	—	1.4370
III-c-IV	Residue	0.218	Lt. yellow	18-22.5	201.2	87.3	1.4582

Fraction III-a-I may be identified as palmitic acid containing a small amount of some acids of higher molecular weight. Fraction III-b was also palmitic acid containing some unsaturated acid. Paper chromatography revealed spots corresponding to palmitic acid for both of these fractions.

Fraction III-c-I was a complex mixture and was not investigated further. Fraction III-c-III was a mixture of octadecanoic acid with an unidentified higher fatty acid. Paper chromatography revealed two spots, one corresponding to octadecanoic acid (R_F 0.72, 0.73), and the other to palmitic acid (R_F 0.45, 0.47) (90% methyl alcohol development). Fraction III-c-IV was an almost pure octadecanoic acid, apparently oleic. A 90% methyl alcohol development of the paper chromatogram revealed a spot with an R_F of 0.85, while a 75% methyl alcohol development revealed a spot with an R_F of 0.42.

Investigation of Fraction IV

A precipitate weighing 0.459 g (Fraction IV-a) was obtained upon the low-temperature crystallization of the methyl esters of the higher fatty acids of Fraction IV from a 3% acetone solution at −16 −20°. This substance was a light yellow, amorphous mass, hard at +20°. The evaporated filtrate yielded 1.014 g of brown substance, semi-liquid at +20° (Fraction IV-b). Subsequent saponification and the usual treatment liberated the free fatty acids from each fraction. Their properties are given in Table 9.

TABLE 9

Properties of Higher Fatty Acids of Fraction IV

Fraction	Weight (g)	Color	Consistency at +20°	Neutralization No.	Iodine No.
IV-a	0.332	Light yellow	Hard	158.6	19.6
IV-b	0.847	Light brown	Soft	163.4	54.6

Fraction IV-a was then further analyzed by low-temperature crystallization from a 1.4% acetone solution. Fraction IV-a-II was further fractionated by low-temperature crystallization from a 3% acetone solution. Fraction IV-b was also recrystallized at low temperature from a 5% acetone solution. A nearly pure eicosanoic acid was isolated from Fraction IV-b-III as a result of an additional low-temperature crystallization from an 8% acetone solution. The properties of the obtained fractions are given in Table 10.

TABLE 10

Properties of Higher Fatty Acids Obtained by Low-Temperature Crystallization of Fractions IV-a and IV-b

Fraction	Crystallization temperature of the residue (°C)	Weight (g)	Color	M.p. (°C)	Neutralization No.	Iodine No.	Refractive index, n_D^{70}
IV-a-I	−5 −10	0.029	Lt. yellow	30.5-31.5	39.6	–	1.4544
IV-a-II	Residue	0.152	White	50-50.5	–	–	–
IV-a-III	−13 −15	0.067	"	51.8-52.3	166.3	8.5	1.4371
IV-a-IV	Residue	0.074	"	50-51.8	183.5	24.4	–
IV-b-I	+5 −5	0.054	Lt. yellow	18.5-21.5	60.0	–	1.4572
IV-b-II	−20 −22	0.063	"	42.5-44.5	114.3	–	–
IV-b-III	Residue	0.518	Yellow	40-42.5	–	–	–
IV-b-IV	0 −10	0.106	"	47-49	177.1	34.3	1.4395
IV-b-V	−10 −20	0.108	"	45-45.8	174.4	43.7	1.4412
IV-b-VI	Residue	0.205	"	28-31	181.5	73.0	1.4444

The physical and chemical properties of Fraction IV-a-I identified it as a neutral substance of an unknown nature, containing a certain amount of fatty acids. Fraction IV-a-III was an unidentified saturated fatty acid containing some unsaturated substance, apparently of a neutral nature, and already detected in the preceding fraction. Only one spot (R_F 0.27, 0.28) was developed by 90% methyl alcohol on the paper chroma-

togram. Fraction IV-b-I contained a large amount of a neutral substance and some unknown fatty acid. Upon development of the paper chromatogram with 90% methyl alcohol part of the substance remained on the place of application as a white spot, while the other part gave a spot, characteristic of fatty acids, with an R_F of 0.77, 0.80. Fraction IV-b-II also contained a significant amount of the above-mentioned neutral substance in combination with a fatty acid which produced a spot upon 90% methyl alcohol development of the paper chromatogram with an R_F of 0.24, 0.25. Fraction IV-b-IV was a mixture of eicosanoic acid with two unknown higher fatty acids. Paper chromatography (90% methyl alcohol development) revealed three spots; one belonged to eicosanoic acid, R_F 0.73, 0.75; the second had an R_F of 0.56, 0.60 and the third an R_F of 0.39, 0.43. Fraction IV-b-V was a mixture of eicosanoic acid with other acids. Fraction IV-b-VI was a nearly pure eicosanoic acid. Paper chromatography (90% methyl alcohol) revealed only one spot, with an R_F of 0.71, 0.73, 0.73. Upon development with 75% methyl alcohol a spot with an R_F of 0.16, 0.19, 0.22 appears.

Investigation of the Undistilled Portion of the Higher Fatty Acids

The undistilled portion of the methyl esters of the high molecular weight fatty acids was divided into 9 fractions after recovery from the fractionating column by several low-temperature crystallizations from acetone (Table 11). Fractions V - IX were obtained by low-temperature crystallization from a 3% acetone solution. Fraction X was obtained by low-temperature crystallization of the residue left over from the preceeding separation from an 8% acetone solution. Fraction XI was obtained by crystallization of the residue from an 11% solution, while Fraction XII was obtained from the residue of the preceeding separation by crystallization from 13% acetone solution. The last residue was Fraction XIII.

TABLE 11

Low-Temperature Crystallization of the Undistilled Portion of the Methyl Esters of the Higher Fatty Acids

Fraction	Crystallization temperature of the residue (°C)	Weight (g)	Appearance of fraction	Saponification No.	Iodine No.
V	+29	0.667	Lt. brown hard mass	26.1	8.1
VI	+9	0.122	"	—	—
VII	−5	0.824	"	90.5	28.5
VIII	−12	2.812	Brown hard mass	108.8	17.5
IX	−25	0.420	"	119.6	26.9
X	−21	0.379	"	122.5	39.8
XI	−24	0.298	"	136.4	55.2
XII	−26	0.305	Brown, paste-like mass	150.1	58.9
XIII	Residue	1.544	"	148.0	64.0

The largest fractions, VIII and XIII, were investigated in detail, while only certain constants were determined in the other fractions. Fractions VIII and XIII were further investigated by distillation in a packing-free microcolumn with an internal tube diameter of 8 mm described by Bower and Cooke [21].

Investigation of Fraction VIII

2.42 g of this fraction was distilled in a microcolumn under a vacuum of 0.2-0.3 mm Hg. The results are given in Table 12.

The free fatty acids were obtained in the usual way from the methyl ester fractions. Their properties are given in Table 13.

It was impossible to investigate in detail the properties of the obtained fatty acids due to the minuteness of Fraction VIII-a. However, the neutralization number indicates that the fraction contains acids with 20 carbon atoms per molecule.

The properties (amorphousness, extended melting range, low neutralization number) of Fractions VIII-b and VIII-c identify them as mixtures of higher fatty acids containing a certain amount of unknown neutral substance.

TABLE 12

Fractionation of the Methyl Esters of the Higher Fatty Acids of Fraction VIII

Fraction	Temperature of the paraffin bath (°C)	Temperature of the insulating mantle (°C)	Weight (g)	Appearance of the fraction at +20°C
VIII-a	160-185	120-150	0.076	Light yellow oil
VIII-b	185-230	150-200	0.423	Light yellow hard mass
VIII-c	230-235	190-210	0.800	" "
VIII-d	230-250	200-210	0.745	" "
VIII-e	230-255	205-230	0.312	" "
VIII-f	Distillation residue		0.060	Hard, brown mass

TABLE 13

Properties of the Free Higher Fatty Acids of Fraction VIII.

Fraction	Weight (g)	Appearance at +20°	M.p. (°C)	Neutralization No.	Iodine No.	Index of refraction n_D^{70}
VIII-a	0.033	Lt. yellow crystalline mass	50-50.5	176.3	–	–
VIII-b	0.386	Lt. yellow amorphous mass	52-58	102.3	24.6	1.4475
VIII-c	0.756	Lt. brown amorphous mass	65.5-69.0	109.7	13.6	–
VIII-d	0.694	"	67.8-69	113.8	12.0	–
VIII-e	0.280	Lt. yellow amorphous mass	45-60	113.9	20.0	1.4490

The separation of high molecular fatty acids from the neutral substance in the VIII-c and VIII-d fractions was effected with the aid of barium salts. 1.1 g of the mixture of both combined fractions was dissolved in 50 ml of 98% ethyl alcohol, the solution was then neutralized by 0.1 N solution of KOH in ethyl alcohol (phenolphthalein). Then 1.5 g of barium acetate dissolved in 10 ml of water was added with agitation. A white voluminous precipitate appeared, which was filtered out after being allowed to settle for 1 hour. A light brown, amorphous substance weighing 0.168 g, with m.p. 51-62° and a refractive index (n_D^{85}) of 1.4540, was obtained from the evaporated filtrate. The residue was washed with 40 ml 96% ethyl alcohol, 150 ml warm H_2O, 40 ml 96% ethyl alcohol again, then dissolved in 40 ml boiling ethyl ether and filtered. The ether solution of barium soaps was treated with a 10% solution of HCl and the free acids were obtained in the usual way. A further separation of the fatty acids was obtained by low-temperature crystallization from a 1.8% acetone solution. The first precipitate appeared at a temperature of 7° (Fraction VIII-cd-I). It was a pale yellow amorphous mass, weighing 0.393 g, having a m.p. 73-74°, a neutralization number of 108.9, an iodine number of 8.5, and a refractive index , n_D^{75}, 1.4455. $[\alpha]_D^{20}$ + 6.16 (in chloroform).

The second precipitate (Fraction VIII-cd-II) appeared at a temperature of −4°. It was a pale yellow crystalline mass weighing 0.318 g, with a m.p. 70-71°, a neutralization number of 113.3, an iodine number of 7.6, an acetyl number of 119.2, 114.0, and a refractive index, n_D^{75}, 1.4454, $[\alpha]_D^{20}$ + 7.26° (in chloroform). The above data indicate that Fraction VIII-cd-II was a nearly pure hydroxy acid having 32 carbon atoms per molecule and 1 hydroxyl group ($C_{32}H_{64}O_3$). The low iodine number is probably due to the presence of a certain amount of analogous unsaturated acid (as in Fraction XIII-d). The structure of this hydroxy acid is probably branched, as it has a comparatively low melting point (70-71°). It dissolves readily in chloroform, benzene, acetone, ether, warm petroleum ether and hot ethyl alcohol. Water is not a good solvent for the potassium salt of this acid, while ether is. This free acid crystallizes from ethyl alcohol in small crystals having a characteristic star shape (see the microphotograph).

Microphotograph of isolated corynomycolic acid crystals.

An acid with analogous properties has been described by Lederer and Pudles [11] and named corynomycolic acid by them. They demonstrated [11,12] the structure of corynomycolic acid to be the following:

$$CH_3-(CH_2)_{14}-CHOH-\underset{\displaystyle C_{14}H_{29}}{\underset{|}{CH}}-COOH$$

It must be pointed out that the α-hydroxycorynic acid described by Gubarev and his co-workers [9] is very similar in its properties to corynomycolic acid. Apparently Fractions VIII-cd-I and VIII-e also represent corynomycolic acid containing some impurities.

The residue from the low-temperature crystallization (Fraction VIII-cd-III) was a brown amorphous substance, weighing 0.160 g, with m.p. 39-40°, a neutralization number of 121.4, and an iodine number of 31.6. The fraction represents a mixture of various fatty acids which was not analyzed in detail.

Analysis of Fraction XIII

1,300 g of this substance was placed in the microcolumn and distilled under vacuum (0.2-0.3 mm Hg). The results are given in Table 14.

The free higher fatty acids were obtained from the methyl esters of the obtained fractions by saponification and decomposition of the soaps by usual methods. Their properties are presented in Table 15.

Fraction XIII-a was a mixture of higher fatty acids containing some neutral substance. Fraction XIII-b was a mixture of saturated and unsaturated acids having, on the basis of its neutralization number, 18-20 carbon atoms in its molecule. Fraction XIII-c was a mixture of higher fatty acids which was not investigated in detail.

Fraction XIII-d was a mixture of high molecular fatty acids having similar molecular weights. It is significant that the methyl esters of the acids in this fraction distilled under the same conditions as the methyl ester of corynomycolic acid. The potassium of the fatty acids in this fraction were slightly soluble in water and highly soluble in ethyl ether. The fatty acids of this fraction were optically active, $[\alpha]_D^{20}$ + 4.15° (in chloroform). This indicated the presence of asymmetric carbon atoms in their molecules. The acetyl num-

ber of this fraction was 122.0 and 125.1. The average molecular weight of the fatty acids of this fraction on the basis of the neutralization number was 481.2. The next fraction, XIII-e, was very similar in constants to the one just described.

Due to the small amount of substance left in this fraction, we were unable to purify it further and cannot give the accurate formulas of the acids contained in it. Apparently there were some hydroxy acids with 31-32 carbon atoms per molecule. The relatively high iodine numbers of these fractions indicated that in addition to monoethylenic acid there was a significant amount of less saturated acids present. It is possible that these fractions contained some corynomycolic acid ($C_{32}H_{62}O_3$) which was isolated in the pure state by Lederer and Pudles [12,13] from the lipids of diphtheria bacilli.

TABLE 14

Fractionation of the Methyl Esters of the Higher Fatty Acids of Fraction XIII

Fraction	Temperature of paraffin bath (°C)	Temperature of the insulating mantle (°C)	Weight (g)	Appearance of fraction at +20°C
XIII-a	140-150	120-130	0.068	Light yellow, liquid substance, with crystals
XIII-b	150-180	130-140	0.150	Light yellow, hard substance
XIII-c	180-210	140-170	0.146	Yellow-brown, honey-like substance
XIII-d	210-240	170-205	0.388	Red, liquid substance
XIII-e	210-240	180-205	0.195	Red, liquid substance
XIII-f	225-255	210-230	0.081	Brown, thick substance
XIII-g	Distillation residue		0.145	Brown, thick substance

TABLE 15

Properties of the Free Higher Fatty Acids of Fraction XIII

Fraction	Weight (g)	Appearance at +20°C	M.p. (°C)	Neutralization No.	Iodine No.	Index of refraction, n_D^{70}
XIII-a	0.059	Light yellow crystalline mass	45-47	166.5	51.0	1.4436
XIII-b	0.130	Light yellow crystalline mass	45.5-47.5	191.0	51.1	–
XIII-c	0.133	Brown, thick oil	–	144.6	58.1	–
XIII-d	0.360	Dark red, light oil	11-13	116.6	69.7	1.4712
XIII-e	0.153	Brown, light oil	–	114.8	70.1	1.4750
XIII-f	0.071	Brown, thick oil	–	110.7	72.9	

SUMMARY

The mixture of higher fatty acids obtained from diphtheria bacilli lipids has a complex composition and contains, in addition to acids, substances of a neutral nature.

In addition to palmitoleic and palmitic acid, octadecanoic (oleic) $C_{18}H_{34}O_2$ and eicosanoic $C_{20}H_{38}O_2$ acids are present in the distillate of higher fatty acids. The unknown higher fatty acids detected by paper chromatography in the Fractions II, III, and IV are possibly branched acids containing 18-20 carbon atoms per molecule.

The presence of corynomycolic acid in the high molecular fraction was established. A fraction of unsaturated high molecular hydroxy acids containing 31-32 carbon atoms per molecule was isolated.

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THE RATE OF DEUTERIUM PENETRATION INTO PLANT TISSUE

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In spite of the large number of published works on the influence of heavy water (deuterium oxide) on plant organisms, the rate of penetration of deuterium into plant tissues has not yet been investigated. We performed a series of experiments with elodea (Elodea canadensis) in nutrient solution containing deuterium in order to investigate this problem and the possible relationship between the rate of penetration and a number of external conditions (deuterium concentration in the outside media, the illumination, the pH of the media, and the temperature).

At the end of each experiment the nutrient solution was discarded, and the elodea plants were rinsed several times in tap water to remove the heavy water present on the surface. After superficial drying on a piece of filter paper the plants were dried in vacuum in a nitrogen atmosphere on a water bath for 2 hours. On the basis of previous experiments all the cell water (87-92% by weight) was collected by liquid air freezing. The water samples were carefully purified according to Rittenberg and Schoenheimer [1] with the following modification: boiling with chromic anhydride for 30 minutes instead of 1 minute, and boiling with potassium permanganate for 15 minutes instead of 3 minutes.

A subsequent purification and interferometric measurements always gave coinciding results, which indicated the total oxidation of organic impurities. The determinations showed a mean deviation of 1%.

TABLE 1

Deuterium Concentration in Cell Water and Surrounding Medium at 18°.

Time in hours	2		24		72		144	
% D, initial	% D, in the experiment	As % of the initial content	% D, in the experiment	As % of the initial content	% D, in the experiment	As % of the initial content	% D, in the experiment	As % of the initial content
16.8	8.5	51	8.9	53	8.4	50	8.5	51
26.8	13.2	49	13.4	50	13.7	51	14.5	54
46.2	23.1	50	22.0	48	24.8	54	23.1	50
59.0	30.2	51	29.5	50	28.9	49	29.5	50

The first experiment shown in Table 1 uncovered two interesting facts: the relative deuterium concentration in the cell water of all specimens was equal approximately to 50%, independently of the initial deuterium concentration of the nutrient solution; secondly, an equilibrium between the deuterium concentration in cell water and in the surrounding medium was established after two hours, even when these concentrations were unequal.

It is interesting that the presence of such an equilibrium did not mean that the deuterium concentration remained rigidly constant, since such fluctuations were greater than the experimental inaccuracies. At the same time, the fluctuations were identical among experiments with various deuterium concentrations, in comparison to fluctuations that were observed in experiments with the same concentration but carried out at different time intervals. During lengthy experiments the great difference is characteristic. This excluded the influence of the variable deuterium concentrations and indicated an equilibrium relationship with the total (physiological) state of the plant (see also Table 2).

No significant results were obtained during the attempt to investigate the influence of light on the rate of penetration (Table 2). The data in Tables 2 and 3 give only relative figures for the deuterium concentration (the deuterium content of the nutrient solution was equal to 100%).

TABLE 2

Deuterium Penetration in Light and Darkness, at 18° C; Initial Concentration of D: 29.5%.

Time (hours)	Light	Darkness
2	52	53
5	50	49
24	51	52

TABLE 3

Influence of Temperature on the Penetration Rate of Deuterium; Initial Concentration of D: 32.1%.

Time (minutes)	18°	5°	Time (minutes)	18°	5°
5	14	18	60	38	48
10	24	–	240	39	49
20	26	33	480	38	46

Recently some data was published on the penetration rate of tritium into bean cells [2] which demonstrated the existence of a similar equilibrium, but an unequal distribution of tritium between the cell water and the surrounding media (45-65%). In these experiments the equilibrium was reached in a much longer period of time (12 hours).

Our subsequent experiments were with short exposures, in order to determine the rate at which the equilibrium would be reached at various pH values and temperatures. A rise in alkalinity from pH 7 to pH 10 did not influence the rate of equilibrium, but a change in temperature from 18° to 5° led to a noticeable change, shown in Table 3 and Fig. 1 (Curves 1 and 2).

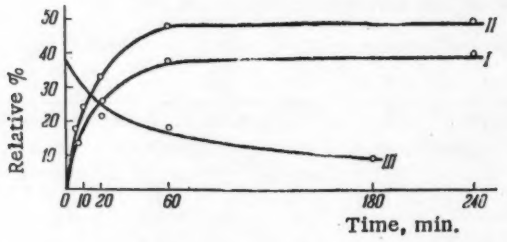

Figure 1. Influence of temperature on the penetration rate of deuterium: (I – at 18°; II – at 5°) and rate of deuterium escape upon transfer of the plant from heavy water into tap water (III).

This data shows that equilibrium was reached after 1 hour and that the equilibrium concentration in this series of experiments at 18° differed from the equilibrium concentration observed during the first experiments carried out for a different length of time but at the same temperature. This might have been the result of a

different physiological state of the plant.

It must be pointed out that exposures of less than 5 minutes duration are not very accurate. The inaccuracy is due to the fact that the deuterium is washed off during the rinsing. Experiments showed that the transfer of elodea, after establishment of equilibrium, from heavy water into tap water for 20, 60 and 180 minutes led to a corresponding loss of 44, 54 and 75% of deuterium from the cell water (see Curve 3 on Fig. 1). If we consider that the penetration rate and the rate at which the deuterium is washed off are equal during the first minutes, it is possible to calculate that during a 10 second rinsing in water the mean deviation in experiments with 5, 3 and 1 minute exposures will be equal to 3, 5 and 15%. Since a relatively large amount of water is retained on the surface of plants, an insufficient rinsing (less than 10 seconds) may also affect the results.

SUMMARY

A dynamic equilibrium was found between the deuterium concentration of cell water and that of the water in the surrounding medium. The deuterium content of cell water is approximately one half that of the surrounding medium and does not depend on the illumination. The rate of deuterium penetration and the equilibrium concentration depend on the temperature, and do not depend on the deuterium concentration or the pH of the medium.

The analogy between the rate of deuterium and tritium penetration into plant cells indicates the similarity of the behavior of both isotopes, previously shown by experiments on animals [3].

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THE MECHANISM OF PROTEIN DENATURATION. NEW EVIDENCE ON THE ABRUPT CHARACTER OF GLOBULAR PROTEIN DENATURATION

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It has been previously shown [1] that protein denaturation occurs in a sudden, abrupt way, in the sense that its rate is extremely fast and that no intermediate compounds can be detected by the usual analytical methods.

All the evidence accumulated to date is based on the correlation of the amount of denatured protein with all the other changes occurring in the solution during the process of denaturation. However, the problem would be settled if it were possible to demonstrate directly without separating the reacting components that both native and denatured protein molecules are really present during partial denaturation of simple proteins. We also had to disregard the assumption that all albumin molecules, in a urea solution for instance, are found in some intermediate state, and that when urea is extracted some of them revert to the native state and others precipitate out as a coagulate.

It was also necessary to obtain conclusive evidence on the state of that part of the albumin which (during partial denaturation) remained undenatured after treatment with urea, heat, etc. It was necessary to reconsider the data of Neurath and Saum [2] who found on the basis of diffusion measurements, that serum albumin molecules in urea solutions of increasing concentrations were in all cases simple proteins. These results may be considered as the main experimental evidence against the concept of sudden denaturation. It was also desirable to extend the number of investigated proteins.

These were the aims of our investigations. Moreover, our work was necessitated by the continuous publishing of investigations which insistingly demonstrate the gradual character of protein denaturation thereby refuting the abruptness of this reaction [3,4 and others].

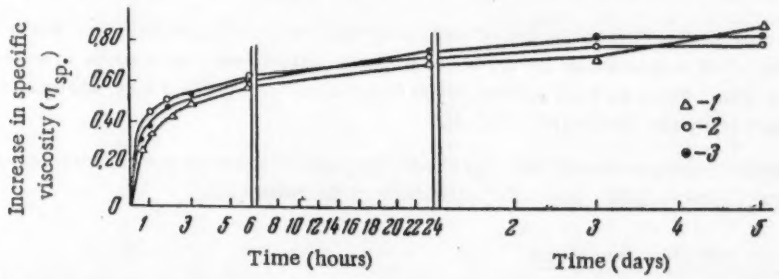

Fig. 1. Changes in the properties of an edestin solution upon its denaturation by urea.
1) Specific viscosity (in η_{sp}); 2) amount of undissolved albumin (in η_{sp}); 3) optical rotation (in η_{sp})

The following proteins were used in our experiments: dry commercial preparation of human blood

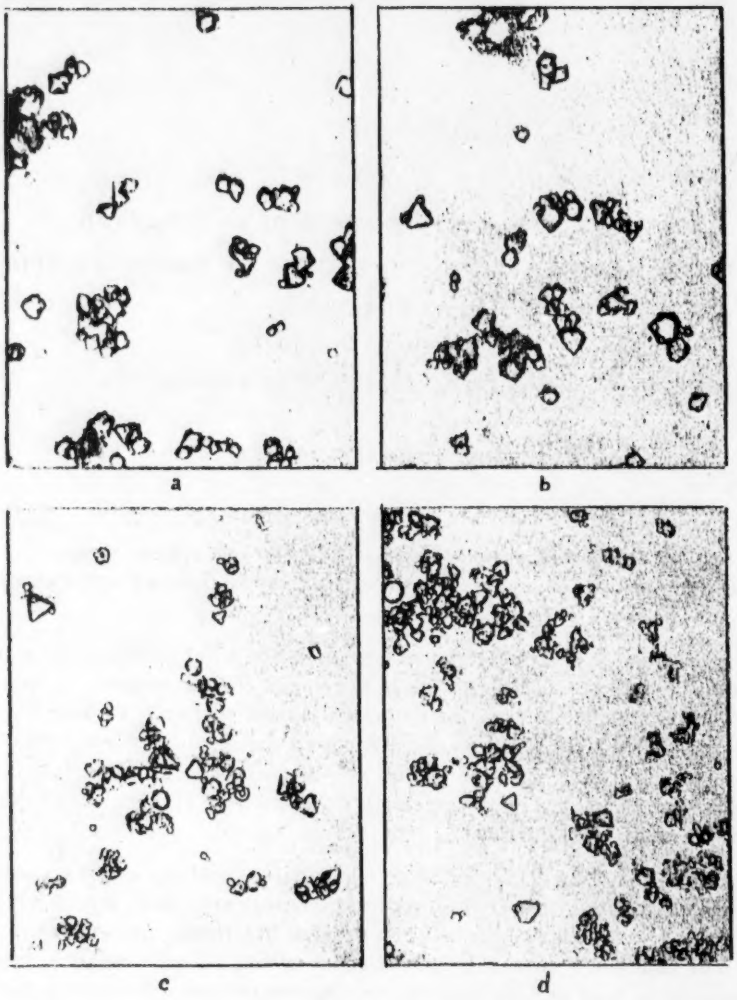

Fig. 2. Crystals of native edestin. Initial preparation - a; undenatured part, crystallized after partial denaturation by heat - b; with urea - c; with potassium rhodanate - d.

serum albumin, re-crystallized 3 times according to Baranovsky, egg albumin, and crystalline edestin, extracted from crushed hemp seed by a 5% solution of NaCl (pH ~ 6) and recrystallized by cooling.

Ordinary, chemically-pure preparations of urea, potassium rhodanate, pyridine, and NaOH were used as denaturing agents. Urea was added in the dry state, while the other agents were added in water solutions. During experiments with edestin we used acetate buffer (most often with a pH of 5.6), while with serum and egg albumins we used phosphate buffer (pH 7.2-7.4).

The electrophoretic measurements were made on the apparatus made in the experimental shop of the Academy of Medical Science, USSR, under the supervision of Rozenberg [5].

Denaturation of Edestin by Urea

In the first series of experiments we investigated the process of formation of denatured protein in relation to other changes in theedestin-urea system. It was first necessary to prepare a solution from which the denatured protein could be quantitatively separated from the native protein. The following solution was found

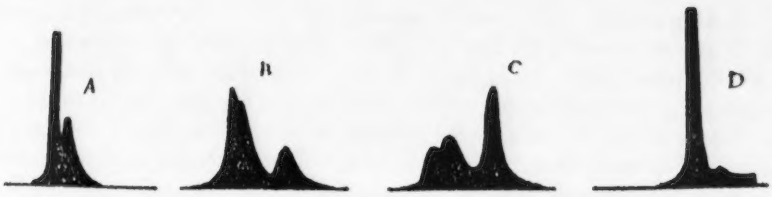

Fig. 3. Electrophoretic investigations of the denaturation of egg albumin in urea solution. Albumin concentration 2.33%; 0.05 M phosphate buffer, pH 7.2; urea concentration 400 mg/ml; duration of electrophoresis: 180 minutes; potential gradient: 2.5 V/cm.
A) Native (initial) egg albumin; B) egg albumin, 30% denatured in urea solution; C) the same, 47.9% denatured; D) the same, 95-100% denatured.

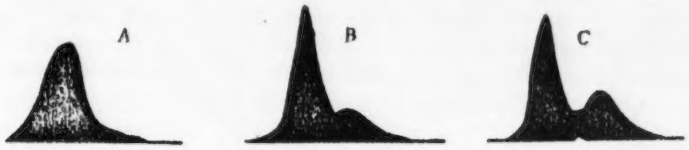

Fig. 4. Electrophoresis of partially denatured serum albumin in urea solution. Albumin concentration 2.5%; 0.05 M phosphate buffer, pH 7.2; potential gradient 2.4-2.5 V/cm; duration of electrophoresis: A) 300 min; B) 150 min; C) 180 min.
A) Native (initial) serum albumin; B) partially denatured serum albumin in urea solution (3 M urea, 8 hr. denaturation at 21°C); C) the same, (2 M urea, 8 hr. 15 min. denaturation at 37°C).

to satisfy the above requirement for edestin: to 1 ml of protein-urea mixture we added 9 ml of a 10% NaCl solution in 0.1 M acetate buffer (pH 5.6); the mixture was left at room temperature for 2-3 hours, the denatured protein was then separated by centrifugation and, after washing with the same denaturing mixture, was dissolved in 3% NaOH; we then performed the biuret reaction as usual. The separation of native and denatured albumin was quantitative, as shown by the curves of Fig. 1.

We used urea as the denaturing agent of edestin. Dry urea, in the proportion of 300 mg per 1 ml of protein was added to a 2.5-3.0% solution of protein in the same salt mixture which was used for the isolation of denatured edestin (a 10% NaCl solution in 0.1 M acetate buffer, pH 5.6). The denaturation was carried out at a temperature of 20-21°C, and control samples were taken at various time intervals. Viscosity and refractive index changes were simultaneously observed at the same temperature in portions of the samples.

A typical experiment is presented on Fig. 1. Without repeating our previous discussion [1] we will point out that the observed changes (during the length of the whole process) coincide quantitatively with the formation of denatured protein and again demonstrate the abruptness of the denaturation process, in this case, of a characteristic globulin: the growth protein edestin, the properties of which are quite different from the previously investigated egg and serum albumins.

State of the Protein Remaining Undenatured After Partial Denaturation

In these experiments we attempted to demonstrate the native state of that part of the protein which remained undenatured throughout the process. Strachitsky and Plotnikov [6] investigated the undenatured protein extracted from an egg albumin-urea system. Having determined that it has a relative viscosity, a specific optical rotation, and that it contains SH-groups, identical to those of the initial protein, these authors concluded that this fraction contained native protein molecules.

In order to demonstrate the native state of the undenatured fraction we used its ability to crystallize which, as is well known, is one of the most characteristic indications of the native state of the proteins. We used crystalline edestin, which undergoes a non-reversible denaturation.

Removal of the denaturating agent from the system (for instance, urea by dialysis) prevents renaturation. Renaturation, therefore, did not occur and the observed phenomena could not have been altered by it.

We added the following amounts of denaturing agents to 15 ml of a 3-4% solution of edestin in a 10% NaCl solution (as usual, in 0.1 M acetate buffer, pH 5.6), 4.5 g of urea (300 mg per 1 ml of protein), or 0.5 ml of a 6% NaOH solution, or 0.8 ml of pure pyridine, or an equal volume of a 25-35% solution of potassium rhodanate. The mixture was left at room temperature and after a definite time interval samples were taken for the crystallization of native protein. In order to extract the undenatured fraction, the sample was diluted 5-8 times by a solution which is usually used for the precipitation of denatured edestin, and left overnight at room temperature. The coagulate was separated by centrifugation or filtration. The filtrate, containing the undenatured protein, was dialyzed against water through cellophane or parchment. The obtained crystals were observed microscopically and compared with crystals of native edestin which had simultaneously undergone the same treatment.

We must recall that edestin forms crystals of widely varying shapes [7]. In addition, the mixture usually contains many crystals of a mixed form. The above observations were made during the separation of native protein in the crystalline state as well as during the separation of that part of the protein which remained undenatured upon partial denaturation with various agents: urea, heat, alkali, potassium rhodanate and pyridine. If the denaturating agent is removed at any time during the process, the part of the edestin remaining native may be crystallized, while the denatured edestin precipitates simultaneously as a coagulate. Many preparations were obtained and analyzed. While our photographs (Fig. 2) do not always illustrate this point clearly (various crystal dimensions) we came to the categorical conclusion that all of the obtained crystalline materials are identical to native edestin (Fig. 2a) and therefore, identical to each other (Fig. 2, b-d). Such samples were also taken at various stages of the protein denaturation. The results were in all cases identical. Therefore, the protein remaining undenatured (by various agents) at all times of the process represents the same edestin.

The most descriptive experiment proved to be the one in which we succeeded in separating, during partial denaturation, the denatured protein (as a coagulate) from the native protein (in crystalline form). Crystals of native edestin precipitate out together with the amorphous denatured protein if at any moment of the process the urea and salt are dialyzed. It is clearly seen that the system contains two types of molecules: those of the amorphous flocculate and those of the native protein. The latter is easily extracted from the mixture by a 10% solution of NaCl, in which the coagulate is absolutely insoluble, and recrystallized.

Separation of a Mixture of Native and Denatured Protein at Various Temperatures

The next series of experiments dealt with the hypothesis of some authors [8] that renaturation occurs upon the removal of the denaturating agent (urea) from the solution of partially denatured protein. According to these authors all the protein molecules in the urea solution are in some intermediate form between the denatured and native state. When urea is removed, part of the molecules returns to the native state while the remainder settles down being irreversibly denatured. Separation of the native from the denatured part in this case, always involves renaturation.

Our experiments on electrophoresis and denaturation in the presence of ferricyanide have directly demonstrated that two types of protein molecules are present in the total mixture during partial denaturation. This is not due to the removal of urea or the renaturation of some molecules, but is the result of an extremely fast and abrupt denaturation change. The assumption of renaturation may also be refuted in another way.

Protein renaturation usually depends on the temperature of the reaction [9-11 and others]. If renaturation would occur at the time of separation of native and denatured protein upon the removal of urea, then the amount of both forms might be regulated by temperature changes. Then, their usual separation by isoelectric precipitation would yield amounts of denatured and native protein (during urea removal by dissolution), depending on the temperature conditions. We performed such an experiment at the following temperatures: 1, 11, 21, 31, and 41°C.

Dry urea was added to a solution of crystalline egg albumin, and the sample was heated on a water bath in such a way as to provoke a 30, 50, or 60% denaturation. Five test tubes with 1 ml each of the above mixture were kept at the following temperatures: 1, 11, 21, 31 and 41°C in a water bath. Five test tubes with 9 ml each of the following denaturing agent for egg albumin: a 10% solution of $(NH_4)_2SO_4$ in 0.1 M acetate buffer, pH 4.7, were kept in the same water bath. When both series of test tubes reached the same temperature, they were mixed. The coagulate was separated by centrifugation and the amount of protein was determined by the biuret reaction.

Table 1 shows that the amounts of native and denatured protein obtained at various temperatures remained constant. We thereby eliminated the hypothesis of renaturation and of the presence of protein molecules in an intermediate state, under the given conditions.

TABLE 1

Separation of Native and Denatured Albumin at Various Temperatures (egg albumin, partially denatured by urea)

Initial albumin concentration(%)	Duration of denaturation(min)	Temperature of denaturation	Extent of denaturation	Amount of denatured albumin, in mg, coagulated during separation of the mixture at the following temperatures:				
				1°	11°	21°	31°	41°
4.0	10	45	36.2	14.4	14.0	14.4	14.6	14.4
4.68	15	50	64.0	31.9	29.3	30.0	30.0	30.0
3.62•	10	55	50.5	18.2	18.2	18.2	19.0	–

•The samples were kept for 2 hours at the above temperatures, before separation of the protein-urea mixture.

Electrophoretic Investigation of Partially Denatured Globular Protein

If we assume that during denaturation only two radically different types of molecules are present in the solution, then their electrophoretic mobility must be different and we should observe two peaks corresponding to both protein states at any moment of the denaturation process.

This assumption is especially valid for solutions in solubilizing substances (urea, etc.). The latter substances do not allow the aggregation of denatured molecules, and therefore prevent the formation of polydispersion aggregates which could have distorted the assumed mechanism. If the denaturation were a gradual, step-like process, then the addition of urea should not provoke the appearance of new electrophoretic components. Under these conditions all protein molecules must be at some denaturation stage and the protein must remain homogeneous. In this case, an increase in the amount of urea must increase the degree of denaturation [2], and under any conditions, only one peak should be observed during electrophoresis.

The appearance in the total mixture of two different electrophoretic components during the denaturation seems to be a direct proof of the abrupt character of this reaction. Some data on this problem have been published [12]. Investigations of partially denatured proteins in urea solutions have still not been carried out, although this material is undoubtedly indispensible for the solution of this problem.

We used egg and serum albumin. The protein was dissolved in 0.05 M phosphate buffer, with pH 7.2, and a known amount of dry, chemically-pure urea was added to a 2-2.5 solution. The buffer used during electrophoresis was the same phosphate solution to which urea was added in the same amount as to the protein. The concentrations of the salts and urea in the buffer and protein solutions were equalized by dialysis before beginning the experiment. The denaturation was performed in a water bath at temperatures of 20 and 37°C. The same urea solution of proteins was used for the observation of the increasing degree of denaturation. The solution containing the protein, buffer and urea was either left at room temperature for various lengths of time or was heated for various times at 37° increasing thereby the degree of denaturation. The latter was controlled as in previous experiments, by the formation of insoluble protein (at the isoelectric point). Samples were taken at various times for electrophoretic controls. All the experimental conditions (such as concentration of protein, buffer, urea, and voltage etc.) were kept constant in order to have comparable results. The potential gradient most often used was 2.4-3.0 V/cm.

Figures 3 and 4 show the results of typical experiments giving direct answers to our problems. Egg albumin denaturation is presented on Fig. 3. The first experiment (Fig. 3, A) represents the initial protein preparation. This confirms once more the previously published observations that crystalline egg albumin is composed of two electrophoretically non-homogeneous components.

The situation radically changed during partial denaturation by urea. We observe on Fig. 3, B a new component advancing slowly behind the fast native protein (both of its peaks are preserved). An increase in denaturation led (Fig. 3, C) to a decrease in native albumin (both of its peaks are still seen) and an increase in the amount of the new slow component. The size of its peak corresponds to the amount of denatured protein. At total denaturation (Fig. 3, D) the native protein peak has completely disappeared, while the denatured (slow) peak has correspondingly increased. The experiment demonstrates the presence of two types of molecules - the native and the denatured - at any moment of the denaturation process.

An even greater interest was presented by the investigation of serum albumin. The initial protein under

our conditions was electrophoretically homogeneous (one peak). Generally, we obtained in this case the same results as with egg albumin under various conditions. The mixture was left at room temperature for 24 hours after addition of urea to the phosphate buffer-protein solution, pH 7.2, up to a final concentration of 2 M, 3 M and more. Under these conditions the partial denaturation was controlled by the formation of insoluble protein.

The electrophoretic investigation demonstrated in all cases the appearance of a new slower component advancing behind the native protein. Its initial peak was thus divided after partial urea denaturation into two peaks, that of the native protein and that of the denatured, in the same way as it occurred in egg albumin. The formation of two peaks was observed in 2 M, 3M and 4M urea solutions. During increasing concentrations of the denaturing agent the size of the slow peak was increasing while the native protein peak was correspondingly decreasing.

Figure 4, A presents the electrophoresis of the initial serum albumin without urea; Fig. 4, B - that of the same protein in 3 M urea (the amount of denaturation was here somewhat smaller than in other experiments); Fig. 4, C - the same protein in 2 M urea, with a slight heating (37°) of the solution for a stronger denaturation. Thus, we observed during partial denaturation of serum albumin in urea solution, as in egg albumin solutions, two various electrophoretic components: that of the native and denatured protein.

Urea Denaturation of Egg Albumin in the Presence of Ferricyanide

The final series of experiments also gave an irrefutable demonstration of the presence of two types of molecules in the solution.

We attempted to find out by direct means whether the SH-groups are liberated in all or only some (the denatured) protein molecules while the remainder (the native) remain inactive in the reaction. For this purpose we investigated the urea denaturation of egg albumin in the presence of ferricyanide.

The following considerations served as a basis for our subsequent experiments. If proteins are partially denatured by urea in the presence of ferricyanide and the necessary conditions for the quantitative oxidation of SH-groups are present in the system, then all the SH-groups liberated during the denaturation process will be oxidized at the moment of their liberation independently from their source (the SH-groups from all the protein molecules denatured gradually, or the SH-groups from some of the molecules denatured abruptly.) If the undenatured part of the protein is subsequently separated by isoelectric precipitation of the denatured part, then the quantitative determination of SH-groups in the undenatured part may give a direct answer to our problem. In the case of a gradual denaturation when all the protein molecules are active, the amount of SH-groups in the native part must decrease according to the degree of denaturation. In case of an abrupt denaturation, when the source of SH-groups will be only a certain group of molecules, the SH-groups in the extracted protein must be that of native egg albumin.

Therefore, if the denaturation is performed in the presence of ferricyanide the latter will "label" those protein molecules which will have (all or only some) free SH-groups. This may be illustrated by the following example. During a 50% denaturation of proteins two possibilities may arise: 1) all the molecules present will undergo changes leading to the liberation of half the SH-groups present, and then the extracted, undenatured part will contain a number of SH-groups 50% smaller; 2) half (50%) of the molecules in the mixture, being denatured, will liberate all their SH-groups, while the remaining molecules, remaining native, will not change their content of SH-groups.

Therefore, the basic problem is to find out whether or not the amount of SH-groups in that part of the protein which remains undenatured during partial denaturation in the presence of ferricyanide, changes. We succeeded in solving this quite clearly.

To 4.0 ml of an 11.6% solution of egg albumin (3 times recrystallized) we added 0.8 ml of 1 M buffer, pH 6.8, 0.8 ml of 0.1 M ferricyanide, and dry urea in the amounts of 425, 500 and 570 mg per 1 ml of mixture. In the following order: urea, buffer, ferricyanide, protein. The mixture was heated in a water bath for 10-15 min. usually at 37°C in order to insure a 40-50% denaturation. The percentage of protein denaturation was then determined by calculating the amount of free SH-groups present in aliquot samples of the mixture. The degree of denaturation was also verified by determining the amount of coagulated protein which, as usual, corresponded exactly to the amount of titrated SH-groups. The salts and urea were then almost completely removed by dialysis through cellophane against redistilled water. To the clear remaining protein solution we then added 1 ml of a mixture consisting of 1.5 ml of a 2 M acetate buffer, pH 4.7, and 0.5 ml of a saturated $(NH_4)_2SO_4$ solution. As

soon as the isoelectric point was reached the denatured part of the protein precipitated out. After one hour, the buffer and the $(NH_4)_2SO_4$ were completely removed by dialysis through cellophane against redistilled water. After centrifugation of the residue we obtained that part of the protein which remained undenatured during partial denaturation. In this mixture we determined the total amount of SH-groups in order to establish whether it changed during the urea treatment in the presence of ferricyanide or whether it remained the same as in the initial (native) protein. During the determination of SH-groups we carried out a complete denaturation, controlling its degree by the method of isoelectric precipitation. 1000 mg of urea per 1 ml of mixture was added and the mixture heated for 1 hour at 37°. Under these conditions the denaturation was complete.

As a result of two dialyses, and the addition of urea and other reagents, the volume of the mixture increased and the concentration of the extracted undenatured protein fraction decreased up to 1.5-3.0%. For the determination of SH-groups we therefore used a 3-4 fold volume. As previously established [13], this significantly increased the accuracy of our determinations. In all cases, a determination of SH-groups in the initial protein, carried out simultaneously under the same conditions, was used as the control. We calculated the experimental error in the determination of SH-groups under the worst conditions (at the lowest protein concentration in the undenatured fraction and the corresponding concentration in the control solution of initial protein). The error amounted to 7-9% of the determined amount of SH-groups and most often to 3-4%. The degree of denaturation was 30-40-50%.

TABLE 2

Urea Denaturation of Egg Albumin in the Presence of Ferricyanide. Content of SH-groups in Albumin Obtained After Removal of the Denatured Part and Conversion to Cysteine.

Amount of SH-groups in the native (initial) albumin (%)	Amount of urea per 1 ml of reaction mixture (mg)	Temperature of denaturation (°C)	Extent of denaturation (%)	Amount of SH-groups in the non-denatured part (%)
0.98-1.09*	425	37	31.2	0.95-1.01**
0.98-1.09*	500	37	41.0	0.97-1.09**
0.98-1.09*	570	39	52.1	0.98-1.00**

*Extreme values obtained after 6 determinations.
**Extreme values obtained after 3 determinations.

Our basic problem was solved with the necessary accuracy. The extracted fraction is identical to native protein. This demonstrates that the changes occurring in a urea solution during partial denaturation are determined only by the abruptly denatured molecules. They are the source of all the liberated SH-groups. Each of the denatured molecules reacts completely. The remaining molecules in the urea solution are native. The assumption of a gradual denaturation of all molecules is thereby refuted, since in this case part of the SH-groups would have been oxidized in all the protein molecules of the system, including those remaining in solution.

DISCUSSION

We obtained during the above experiments direct evidence that at any time of the denaturation process the partly denatured globular protein contains only two types of molecules: native and denatured. We established that during partial hydrolysis the protein molecules are not found in some intermediate state as thought by some authors [2, 3, 8 and others]. We also experimentally refuted the assumption that during the removal of the denaturing agent, part of the protein is renatured (returns to the native state) while the other is completely denatured and precipitates out as a coagulate.

The obtained experimental data indicate that the structural changes of globular protein molecules occurring during denaturation have an abrupt character. It is surprising that some authors, for instance Gaurovitts [14] considered this problem as insoluble due to the absence of the necessary analytical methods. The intermediate forms (between the native and the denatured) are hard to detect by the usual means possibly because of their instability. Preceding the abrupt denaturation is a "gradual and imperceptible accumulation of quantitative changes" in the protein molecule which leads to basic changes in the chain structure.

Our electrophoretic experiments on partly denatured protein in concentrated urea solutions, especially those on serum albumin, refute the data of Neurath and Saum [2] on the monodispersion of protein molecules in urea solutions. These data were obtained on the basis of diffusion measurements. They can probably be explained by the fact that the native and denatured protein molecules in urea solutions have particles with similar diffusion rates. The results of electrophoresis are in complete accord with solubility observations. As established by us previously [15] after denaturation in urea solution of increasing concentration, the egg and serum albumins also separate into two components under the conditions of isoelectric precipitation. Their amounts correspond to the area of the peaks on the elctrophoretic curve.

It is usually conceded that the breaking of a relatively small number of weak bonds [16, 17], approximately 10-20% of their total number, is the basic reason for the occurring changes in the configuration of the peptide chains. The specific chain structure may remain undisturbed until the breaking of the last two or three bonds, after which there follows an abrupt denaturation change in the molecules. It is now clear that the degree of denaturation in a given protein mass does not reflect the amount of changes occurring in each molecule, but indicates a relationship between the denatured and native part. With an increase in denaturing action the amount of denatured protein increases. In all cases (for instance upon temperature increases, increases in urea concentration, etc.) there is a time interval during which the increase is especially rapid. This indicates, as already noted by Pasynsky [18] that the abruptness of the change during denaturation has a dual nature and is the expression of an abrupt change in the structure of individual protein molecules and in the relative amount of denatured molecules.

In order to demonstrate the abruptness of denaturation, it is necessary to investigate individual proteins rather than a system including several proteins [19 and others]. It is also necessary to isolate the denaturation proper from all the preceding and following changes. Without these prerequisites it would be impossible to obtain a clear cut answer to this problem, and in particular to observe a quantitative relationship among the changes with the aid of various methods.

Abruptness undoubtedly characterizes the denaturation observed here. The ability of the giant protein molecule to alter its structure abruptly is of fundamental interest. Its gradual denaturation, the step-like changes occurring at various points of this complex molecule, seemed quite logical. This abruptness is not only a basic concept in the mechanism of denaturation but it also characterizes an important property of the macromolecular structure of globular proteins.

Our views presented above are being constantly supported by new and diversified observations appearing regularly in the literature. Thus, Bulankin and his co-workers [20-22] obtained a number of facts testifying to the abruptness of acid-basic denaturation. The reaction mechanism has been confirmed in new proteins, by the use of new methods such as polarographic analysis and others [25]. Among the works confirming our view we should mention the investigation of Bresler [26] on the abruptness of protein synthesis under pressure and other investigations on the abruptness of protein hydrolysis under the influence of proteolytic enzymes [27, 28 and others].

SUMMARY

Changes in specific viscosity and optical rotation at any moment of the denaturation process of edestin by urea correspond quantitatively to the conversion of protein into its insoluble form.

During partial denaturation of edestin, the remaining undenatured protein may be crystallized. This was established during the action of urea, potassium rhodanate, sodium hydroxide, and pyridine, and denaturation by heat. The form of the crystal corresponds in all cases to that of native edestin crystals. The removal of salts and urea by dialysis leads to the simultaneous precipitation of the denatured protein coagulate and of the native crystals.

The electrophoresis of egg and serum albumins in concentrated urea solutions (without removal of the latter) shows the formation of two moving boundaries corresponding to the native and denatured proteins during partial denaturation. The native boundary is the fastest. With an increase in denaturation the slow component increases at the expense of the faster component. At total denaturation only one peak is present: that of the slow component. These observations completely refute the data of Neurath and Saum [2] on the monodispersion of protein molecules in urea solutions.

During partial urea denaturation of egg albumin in the presence of ferricyanide a part of the protein molecule remaining native (its SH-groups) does not react with ferricyanide. The SH-groups of the remaining part

react completely thereby determining the occurring changes. If during the action of urea the protein molecules were in some intermediate state they would all have reacted with ferricyanide.

The separation of native and denatured protein during partial denaturation, by isoelectric precipitation carried out at various temperatures, demonstrated that the latter do not influence the amounts of both protein fractions obtained after separation. These and other experiments refute the assumption of the "intermediate state" of protein molecules in urea, and on the renaturation of some of them at the moment of urea removal.

The conclusion is that at any time of the denaturation process two types of molecules are present in the solution: the native and the denatured. The intermediate forms (apparently due to their instability) are not detected by the usual methods. Therefore we obtained exhaustive evidence that the structural changes observed in the investigated globular proteins during denaturation occur as a characteristic abrupt conversion.

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THE ROLE OF SULFUR-CONTAINING AMINO ACIDS IN THE VARIATIONS OF URINE N'-METHYLNICOTINAMIDE CAUSED BY THE PROTEIN CONTENT OF THE DIET

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Numerous investigators have shown that the products of nicotinic acid metabolism excreted in the urine will increase with a decrease in the protein content of the diet [1, 2]. The cause of this phenomenon, which demonstrates the relationship between the protein and nicotinic acid metabolisms was not clear. The information obtained from previous experiments performed in our laboratory opened the way for further investigations of this problem [3]. These experiments demonstrated a definite relationship between the metabolism of S-containing amino acids and that of nicotinic acid. One of the manifestations of this relationship is the significant decrease in the amount of N'-methylnicotinamide (MNA) excreted in the urine observed after the addition of large amounts of cystine, cysteine or methionine to the diet (experiments on rats) [3-5]. At the same time, a decrease in the amount of S-containing amino acids in the diet increased the MNA excretion. Further experiments demonstrated that no other amino acid has the ability similarly to influence the excretion of MNA [6]. These facts led to the assumption that the main reason for the observed relationship is a varying content of S-containing amino acids in the diet. The experiments reported here demonstrated the correctness of our assumption and also brought to our attention the possible influence of the relationship between tryptophan and the S-containing amino acid contents of the diet, and nicotinic acid metabolism.

METHODS

The experimental diet was of the following composition: corn starch 23%, sugar 33%, fat 20%, and salt mixture 4% (according to Jonson and Foster [7] with the addition of sodium fluoride and alum)*. 20% of casein was given in order to fulfill the protein requirements. When this amount was reduced to 9%, the amount of starch was correspondingly increased.

Brewer's dried yeast (0.75 g per day containing approximately 0.4 mg of nicotinic acid) was given as the source of vitamin B. The animals also received 20 I. U. per day of vitamin A and 8 I. U. per day of vitamin D. 1-2 mg of vitamin E were given every 7 days.

Food and water were given to the animals ad libitum. All animals were kept in individual cages with perforated bottoms for collection of the excrements. The weight of the animals varied from 140 to 160 g. The N'-methylnicotinamide in the urine was determined by the Huff and Perlzweig method [8]. All rats began to receive at the outset of the experiments a diet containing 20% protein, and the MNA was determined in their urine. Subsequently, some of the animals continued to receive the same diet, while the others were put on a 9% protein diet, to which some S-containing amino acids were added in order to keep their amount equal to the amount received with the 20% casein diet. 58.3 mg of dl-methionine and 6 mg of l-cystine were added daily to the 9% casein diet of each animal. These are the amounts of amino acids contained in the 11% difference in amount of casein which the rats received during one day (the calculations were made according to Blok and Bolling [9]). The urine MNA continued to be determined in the second part of the experiment.

* There were 0.025 mg of aluminum and 0.904 mg of fluoride in 4 g of salt mixture.

RESULTS

The results were identical in the experiments performed on several series of animals. Therefore, the results obtained in only one of these series are presented. As seen in Table 1, the urine MNA noticeably increases after the decrease of protein in the diet to 9%.

TABLE 1

Content of N'-methylnicotinamide (μg) in Daily Urine of Rats Receiving 20% Casein at the Outset of the Experiment and 9% after the 7th Day

Duration of expt., days	1-3	4	5	6	7	8	9	10	11	12	13	14
Rat designation	20% casein				9% casein							
A-1	–	83	86	71	81	63	57	63	117	199	200	235
A-2	–	88	75	91	98	83	35	48	70	121	158	147
A-3	–	159	144	135	148	113	150	231	190	216	375	352
A-4	–	104	113	163	79	89	119	124	147	–	270	249

The experiments involving animals which received additional methionine and cystine gave different results. In this series, the 20% casein diet was reduced on the 7th day to 9%. Addition of methionine and cystine was begun at the same time. As seen in Table 2, the urine MNA began to decrease progressively.

TABLE 2

Content of N'-Methylnicotinamide (μg) of Daily Rat Urine after the 20% Casein Diet Was Replaced by a Mixture of 9% Casein + 58.3 mg of dl-Methionine + 6 mg of l-Cystine

Duration of experiment (days)	1-2	3	4	5	6	7	8	9	10	11	12
Rat designation	20% casein					9% casein + methionine + cystine					
B-1	–	196	298	218	266	236	321	61	71	66	55
B-2	–	235	186	254	299	196	154	40	128	111	77
B-3	–	230	367	460	416	224	108	66	68	59	–
B-4	–	–	170	262	178	115	13	14	37	35	16
B-5	–	117	248	132	175	142	91	27	45	57	77

It is possible that the urine MNA of the second group decreases due to a significant decrease in trypyophan, the source of nicotinic acid which occurs in the presence of a protein deficiency in the diet. Such an assumption must be rejected, however, since our data on the first group of animals show that the urine MNA increases in the presence of a 9% protein diet and of the same tryptophan content.

After the 7th day of the experiment, the diet of the second group of animals differed from that of the first only by its content of methionine and cystine. Our previous experiments [3] have shown that the addition of S-containing amino acids to the diet decreases the excretion of urine MNA in rats. This can be clearly and constantly observed when the amount of proteins in the diet is reduced. At the same time, no other amino acid component of casein does possess this ability [6]. On the basis of the above facts, we may consider that the lower content of urine MNA in animals of the second group as compared to that of the first, while both groups were fed on a 9% protein diet, resulted from the addition of methionine and cystine to the diet of the second group.

The fact that in our experiments the changes in excreted urine MNA were mainly due to methionine and cystine (and not to tryptophan) contents of the diet has also been confirmed by a series of experiments performed by one of us [6] on the addition of single amino acids and mixtures of them to the diet.

In these experiments, a mixture of crystalline amino acids identical in composition and amount to that

which is present in an 11% casein diet, was added to the 9% casein diet at the beginning of the 8th day of the experiment. However, tryptophan was omitted from this mixture (the mixture was prepared according to the data of Blok and Bolling [9]).

The addition of such a mixture to the basic diet resulted in a sharp decrease in the excreted urine MNA; no such decrease was observed when methionine and cystine were omitted from the mixture in addition to tryptophan. Since tryptophan was excluded from the diet of both groups the retention of nicotinic acid in the animals of the first group resulted exclusively from the addition of large amounts of S-containing amino acids to their diet.

The sulfur-containing amino acids serve the purpose of anchors for nicotinic acid. The size of this anchor will determine its ability to retain various amounts of nicotinic acid. Apparently, nicotinic acid is functional in the metabolism of S-containing amino acids. For this reason, the nicotinic acid requirements of the organism are raised in the presence of an increased amount of these amino acids in the diet. When the amount of S-containing amino acids is decreased the organism excretes the surplus of nicotinic acid.

These relationships explain the observations made during the treatment of pellagra [10]. When large amounts of white cheese were given to pellagra patients the excretion of urine MNA was at first completely stopped, regardless of the fact that large amounts of tryptophan were present in the white cheese. The excretion of MNA resumed only after several days. We must assume that this immediate retention of nicotinic acid was due to an increased requirement of the organism resulting from the presence of methionine and cystine in the white cheese.

Since tryptophan is the initial compound in the biosynthesis of nicotinic acid, we assumed that the addition of this amino acid could compensate for the increased nicotinic acid requirements arising from the addition of methionine and cystine to the 9% casein diet and that in this case no decrease in urine MNA should be expected. In order to check the accuracy of our assumption we added to the diet of two rats 30 mg of dl-tryptophan in addition to cystine and methionine. Such an amount of this amino acid is usually present in the daily 11% casein diet. The experiment shows that in this case no decrease in urine MNA could be detected (Table 3).

TABLE 3

Content of N'-Methylnicotinamide (μg) in Daily Rat Urine after 20% Casein Diet Was Replaced by a Mixture of 9% Casein + 58.3 mg of dl-Methionine + 6 mg of l-Cystine + 30 mg of dl-Tryptophan.

Duration of experiment	1-3	4	5	6	7	8	9	10	11	12
Rat designation	20% casein				9% casein + methionine + cystine + tryptophan					
C-1	–	260	266	142	123	153	196	318	382	280
C-2	–	116	112	134	89	106	116	116	174	155

Table 3 shows that tryptophan not only prevented the decrease in MNA excretion but provoked a tendency toward an increased excretion of the latter in the urine. This amount of tryptophan provided for a sufficient and even a slight excess amount of nicotinic acid to meet the requirements of the organism in the presence of S-containing amino acids in the 9% casein diet. Thus, the amount of tryptophan determines the reaction of the organism to the introduction of S-containing amino acids, in as much as this reaction is expressed by the intensity of MNA excretion.

We have already noted that our experiments point to the fact that the nicotinic acid retention, provoked by the S-containing amino acids, is a factor of the utilization of this acid in the metabolism of methionine and cystine. We used a diet containing 9% of proteins (casein) in all our experiments. It has been shown by Osborne and Mendel that a 9% casein diet inhibits the growth of young animals as a result of its exclusive lack of S-containing amino acids and that normal growth is restored upon addition of cystine to the diet. In as much as in these and previous experiments [3] the experimental animals were still in their growth period, the following question may be asked: is nicotinic acid directly involved in the metabolism of S-containing amino acids or does its increased use depend only on the general non-specific intensification of the metabolism resulting from

the intensification of the growth process? In order to answer this question we performed a series of experiments on four adult rats (weighing 260-270 g). These animals received the above described food mixture containing at the beginning 20%, and then 9% of casein. On the 11th day of the experiment, 60 mg of dl-methionine was added to the diet, per day. The results showed that immediately after this addition the urine MNA content decreased as in previous experiments on still growing animals [3]. The four rats gave identical results. Consequently, Table 4 gives the results obtained from only two of these animals.

Therefore, the addition to the diet of S-containing amino acids provokes a retention of nicotinic acid just as intensively as it did in previous experiments [3] and in experiments on adult animals. The necessary conclusion is that the retention of nicotinic acid results from the general non-specific intensification of the metabolism peculiar to growth processes and mostly from the utilization of nicotinic acid in the metabolism of S-containing amino acids.

Since the addition of S-containing amino acids to a 9% casein diet intensifies the growth of young rats [11] we performed an additional series of experiments in which we used a protein-free diet in order to eliminate completely the possibility that the observed increase (Table 1) or retention (Table 2) of MNA is the result of corresponding changes in the general intensification of the metabolism. To this diet we added methionine and other indispensable amino acids. By using a protein-free diet, we eliminated the possible occurrence of those peculiarities of the influence of methionine, in comparison to other amino acids, which occur in the presence of a 9% casein diet.

TABLE 4

Content of N'-Methylnicotinamide (μg) in Daily Urine of Adult Rats, After 60 mg of dl-Methionine Was Added to the 9% Casein Diet

Duration of experiment	1	2	3-7	8	9	10	11	12	13	14	15
Rat designation	20% casein		9% casein				9% casein + 60 mg of methionine				
D-1	–	–	–	304	304	195	334	229	80	99	78
D-2	–	–	–	–	79	179	196	103	73	63	51

These experiments were performed on four groups of rats weighing from 170 to 180 g. The growth processes in these animals were still active. Each group consisted of four rats. For a short time the animals received the same diet with 20% casein as in preceeding series. On the 9th day all proteins were excluded and the diet of the first group (control) remained protein-free for the duration of the experiment. On the 11th day amino acids were added in amounts equal to those contained in a 20% casein diet. Either dl-methionine (106 mg) or lysine (210 mg) or valine (210 mg), were added. The amount of MNA excreted in the urine per day was determined in all animals.

TABLE 5

Content of N'-Methylnicotinamide (μg) in Daily Rat Urine After Elimination of the Dietary Protein and Subsequent Addition of Either Methionine (106 mg), Lysine (210 mg) or Valine (210 mg)

Duration of experiment	1-6	7	8	9	10	11	12	13	14	15
Rat designation	20% casein			Protein eliminated						
E-1	–	314	129	102	87	124	74	87	157	142
E-2	–	285	226	121	61	45	157	108	217	–
				Methionine added						
E-5	–	319	167	219	241	187	196	15	39	–
E-6	–	172	260	135	–	245	185	82	55	74
				Lysine added						
E-9	–	–	324	380	195	235	195	371	196	292
E-10	–	140	149	116	123	89	190	–	146	242
				Valine added						
E-13	–	193	226	154	80	143	68	112	107	166
E-14	–	295	–	380	186	287	277	155	198	219

The results of these experiments primarily demonstrated that during total exclusion of proteins from the diet, the excretion of MNA, which at first decreased, began to increase as it did in the case where the amount of casein in the diet was decreased from 20% to 9%. They also indicated in this case that methionine is the agent which retains nicotinic acid, and that valine and lysine are devoid of this ability, although the latter amino acid has a most important function in the normal growth processes of young rats (Osborne and Mendel [11]). The results obtained in each group on all animals were identical. We, therefore, give in Table 5 the results obtained on only two animals of each group. It becomes apparent that only methionine decreased the MNA content in urine, although all the free amino acids used are essential and were added to the protein free diet.

DISCUSSION

The results of the above described experiments indicate that the increased excretion of MNA in the urine occurring after a decrease or even complete exclusion of proteins from the diet depends on the reduced content of S-containing amino acids in the diet. The metabolism of these amino acids is apparently related to an increased use of nicotinic acid. This also explains the decrease in urine MNA excretion when the content of methionine or cystine in the diet increases [3]. Since a decrease in the protein content of the diet increases the excretion of the products of nicotinic acid metabolism in the urine of rats as well as of dogs [1], which are unable to synthesize nicotinic acid, our results are most probably true for humans. The obliterated development of pellagra in the presence of protein deficiency may probably be explained by a decrease in nicotinic acid requirements occurring during a decrease of the diet protein. Such a form of pellagra was prevalent during the siege of Leningrad [12]. The observations made on pellagra patients may serve as an example of the relationship between the metabolism of nicotinic acid and that of S-containing amino acids in human pathology. The amount of cystine in the nails of such patients significantly decreases regardless of the fact that the total amount of protein remains unchanged [13].

Our results also indicate that an increase in the content of methionine and cystine in the diet decreases the excretion of urine MNA only if the amount of tryptophan present in the diet is limited and does not satisfy the increased nicotinic acid requirement of the organism. Thus, the relative amounts of the S-containing amino acids, tryptophan and nicotinic acid, present in the diet, will determine to a large extent the fate of the nicotinic acid in the organism. Therefore, the content of MNA in urine may give an indirect indication of the amounts of methionine and cystine present in the diet and of the relationship between these two amino acids and tryptophan in the diet. For the time being, such conditions may be held true only in those animals which are able to synthesize nicotinic acid, since our experiments were done on rats.

It is difficult at the present time to propose a definite solution to the problem of nicotinic acid utilization. Is nicotinic acid utilized directly in the metabolism of S-containing amino acids, or is the nicotinic acid requirement raised as a result of the fact that increased amounts of these amino acids intensify the growth, and consequently the total metabolism, of animals in the presence of a relative protein deficiency, as has been shown by Osborne and Mendel [11]? In addition to our results presented in Tables 2, 3, 4 and 5, the discovery made during previous experiments, of the ability of nicotinic acid to inhibit the development of pathological processes arising in the presence of excessive amounts of cystine [3, 5] should be mentioned as supporting the hypothesis of a direct relationship. The ability of tryptophan to prevent the accumulation of liver fat resulting from a high cystine intake [14] supports the same assumption.

There is no doubt that the disruption of the equilibrium between the metabolism of S-containing amino acids and that of nicotinic acid (tryptophan) may result in the development of pathological processes. In fact, the growth of rats is inhibited by the addition of gelatin to a 9% casein diet only if cystine and methionine are added simultaneously; however, normal growth is restored upon addition of nicotinic acid to the diet [15].

These facts confirm the existence of a relationship between the metabolism of S-containing amino acids, nicotinic acid and tryptophan. Further experiments are, however, necessary in order to determine the nature of this relationship. The use of the physiological function of Coenzyme III [16], containing nicotinamide, remains subject to discussion since its very existence cannot yet be considered as definitely proved.

It is difficult at the present time to correlate our observations with those phenomena that occur in the presence of the so-called "equilibrium upset" (imbalance) of amino acids which results in nicotinic acid deficiency and inhibition of animal growth [17]. In any case, the amino acids inhibiting the growth of young rats do not influence the excretion of nicotinic acid derivatives [18] in the urine.

SUMMARY

A decrease in the amount of protein (casein) present in the diet leads to an increase in the excretion of N'-methylnicotinamide in the urine. This is due to a decrease in the amounts of methionine and cystine present in the diet.

The addition of methionine and cystine to a low-protein diet prevents an increase in the excretion of N'-methylnicotinamide in the urine.

Nicotinic acid is active in the metabolism of S-containing amino acids. The ratio of the amounts of S-containing amino acids, tryptophan and nicotinic acid, present in the diet is an important factor determining to a large extent the fate of nicotinic acid within the organism.

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PROTEIN METABOLISM AND OXIDATIVE PROCESSES IN DENERVATED MUSCLES OF HYPOTHYROID ANIMALS

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It has been previously shown by various authors that denervated muscles become hypersensitive to the action of hormones and chemical substances.

The hypersensitivity of denervated structures has been investigated in detail by Kennon and Rozenblyut [1]. The hypersensitivity of muscles to thyroid hormone has been described by Hines and Knowlton [2]. In experiments on rats they demonstrated that amounts of thyroxin, insufficient to lower the glycogen content of muscles of normal extremities, were sufficient to lower it in denervated muscles. Huf and Fischer [3] demonstrated that thyroxin decreased, and methylthiouracil inhibited, atrophy of rat muscles after their denervation. They point to the fact that the biochemical changes occurring in muscle after its denervation are similar to those observed in hyperthyroidism: in both cases the protein metabolism is intensified, the respiration increases, and the glycogen content decreases.

On the basis of these facts we attempted to investigate the protein metabolism and the oxidative processes in denervated muscles of normal and hypothyroid animals in which 6-methylthiouracil (6-MTU) was used to inhibit the function of the thyroid gland. We decided to perform these experiments on the basis of the fact that the thyroid gland is an important link in the neuro-humoral regulation of the oxidative processes and protein metabolism. We evaluated the protein metabolism by observing the changes occurring in proteolytic processes and in the rate of incorporation of labeled methionine. The oxygen absorption by tissues was taken as an indication of the intensity of the oxidation processes. At the same time, we investigated the activity of pyrophosphatase, which increases in muscles during hyperthyroidism [4] and after denervation [5].

EXPERIMENTAL

Methods

The experiments were performed on adult rabbits. The sciatic nerve was severed at the level of the anterior third of the femur of one extremity. The muscles of the other extremity were used as controls. Some rabbits received 100 mg per 1 kg of animal weight of 6-MTU for 30 and more days before the operation; the animals continued to receive 6-MTU after the operation.

The rabbits were decapitated at various periods of time after the denervation, the muscles were excised and cleaned from fascia and tendons in the cold. The gastrocnemius and plantaris muscles were used; the extent of muscular atrophy was judged by weighing the muscle. We report here on the weight of M. plantaris only.

The proteolytic activity was determined by the increase of protein-free nitrogen occurring upon the incubation of muscle pulp in acetate buffer (pH 4) for 6 hours at 38 degrees. The rabbits used for the investigation of the rate of labeled methionine incorporation received the amino acid 17 to 20 hours before the experiment (5000 cpm per 1 g of weight). The muscle proteins were precipitated by 10% trichloroacetic acid, the precipitate washed several times by 5% trichloroacetic acid, then the fats removed by a 20-hour extraction and washed with ether-alcohol (3 to 1), pure ether and dried to a constant weight. The radioactivity was measured by an end counter on 10 mg of dry protein.

The oxygen consumption of muscle pulp was determined in a Warburg apparatus. The mixture contained 200 mg of muscle pulp in phosphate buffer (pH 7.4). The observations lasted 30 minutes. The intensity of respiration was expressed in μl of O_2 absorbed per 1 g of fresh tissue during 30 minutes.

For the determination of pyrophosphatase, 200 mg of tissue was homogenized in the cold with 10 ml of

TABLE 1

The Influence of Denervation on Muscle Atrophy and Rate of Proteolysis

Number of days after denervation	Muscle weight (g)		Change (%)	Increase of residual nitrogen after incubation (mg%)		
	Control	Denervated		Control	Denervated	Change (%)
10	2.40	2.00	− 16.7	113	159	+ 40.7
10	2.80	1.75	− 37.5	114	206	+ 80.7
14	2.82	1.80	− 36.2	102	274	+ 168.6
15	2.50	1.60	− 36.0	113	276	+ 144.2
16	2.40	1.45	− 39.6	100	215	+ 115.0
19	3.70	2.30	− 37.9	91	174	+ 91.2
19	2.30	1.50	− 34.8	89	225	+ 152.8
19	3.90	2.10	− 46.2	83	377	+ 354.2
20	2.75	1.52	− 44.8	71	216	+ 204.2
20	2.75	1.25	− 54.6	91	336	+ 269.2
20	3.05	1.62	− 46.9	98	252	+ 157.1
21	2.20	1.17	− 46.9	87	262	+ 201.1
22	3.45	1.77	− 48.7	74	283	+ 282.4
24	3.37	2.25	− 33.3	132	466	+ 253.0

double distilled water and quartz sand. The experimental mixture contained 0.5 ml of borate buffer, pH 7.4, 1 ml of a solution of sodium pyrophosphate (2 mg), 0.1 ml of 1 M $MgCl_2$ and 1 ml of an aqueous muscle extract. The incubation lasted for 10 minutes in a water bath at 38 degrees. 1 ml of 10% trichloroacetic acid was added to all the test tubes after 10 minutes.

The trichloroacetic acid was added to the control tubes before the addition of the extract; the phosphorus was determined in the filtrate by the colorimetric method of Fiske-Subbarow after protein precipitation.

Results

The data obtained on muscle atrophy, protein composition and rate of incorporation of labeled methionine are given in Tables 1 and 2.

TABLE 2

The Incorporation of Methionine S^{35} by Muscle Proteins (in cpm per 10 mg of dry protein)

Days after denervation	Control muscle	Denervated muscle	Change (%)
10	25	25	0
10	25	24	0
15	32	35	9.3
16	42	51	21.4
18	75	122	62.6
20	48	65	35.4
20	46	109	137.0
20	66	109	65.1
21	29	91	213.8
23	60	110	83.3
23	42	61	45.2
23	30	78	160.0
24	72	126	75.0

TABLE 3

Oxygen Absorption

(in µl per 1 g of muscle, during 30 minutes)

Days after denervation	Control muscle	Denervated muscle	Change (%)
10	101.5	102.0	0
10	185.0	197.0	+ 6.4
15	141.5	154.0	+ 8.8
17	112.7	154.5	+ 37.0
18	85.0	100.0	+ 17.6
19	107.0	118.0	+ 10.2
20	107.5	155.0	+ 44.1
20	87.5	136.0	+ 92.5
20	93.0	98.5	+ 5.9
20	125.5	132.5	+ 5.5
20	90.0	160.0	+ 77.7
21	100.0	150.0	+ 50.0
23	125.0	130.0	+ 4.0
23	137.0	234.0	+ 70.8
27	107.5	118.0	+ 9.7

The increased proteolytic activity observed after denervation is in accordance with the results obtained by other investigators indicating in this case a significant increase of protein decomposition (Table 2).

We report the data on the changes in proteolysis beginning with the 10th day after denervation, although a noticeable increase in protein decomposition occurred on the 5th day. At the same time the muscle began to decrease in weight. An increase in the rate of incorporation of labeled methionine as well as a more intensive protein decomposition were observed from the 15th-16th day after the operation (Table 2).

The data on oxygen absorption in normal and denervated muscle is presented in Table 3.

On the basis of the results we may state that oxygen consumption increases in denervated rabbit muscle. Apparently this increase in oxygen consumption cannot be correlated with the extent of atrophy, since we could not observe any changes in the intensity of respiration as a function of the time lapsed since the denervation.

The prior observations of the increase of pyrophosphatase activity in denervated muscles [5] were confirmed by our experiments. Consequently, we give the results of only some experiments performed during this series (Table 4).

TABLE 4
Pyrophosphatase Activity in Muscles
(μg of phosphorus per 1 ml of extract)

Days after denervation	Control muscle	Denervated muscle	Change (%)
14	26	48	+ 84.6
17	46	117	+ 154.3
19	26	46	+ 77.0
19	32	57	+ 78.1
20	71	142	+ 100.0
20	47	106	+ 125.5
21	41	108	+ 163.4
21	53	120	+ 126.4
21	49	98	+ 100.0

The subsequent investigations on the rate of protein metabolism and oxidative processes were carried out on rabbits which were given 6-MTU as the agent of hypothyroidism. As in previous experiments, the muscles of one extremity were denervated and those of the other served as controls. The data collected during these experiments is presented in Table 5.

As can be seen from Table 5 the muscle atrophy is significantly reduced in the majority of cases after the denervation of the muscles of hypothyroid animals. In 7 out of 19 cases, the atrophy, measured by weighing the muscle, remained the same as in normal rabbits for the same period (13-14 days) after denervation. In the remaining cases the decrease in weight of the denervated muscles was significantly delayed.

The significantly weaker proteolytic activity in denervated muscles of hypothyroid animals must be noted. In some cases we could not observe any substantial difference in the increase of residual nitrogen after incubation of denervated and normal muscles of hypothyroid animals. During the same period (13-27 days), the denervation of muscles in normal animals was accompanied by a significant increase in the proteolytic activity (Table 1).

The difference becomes apparent when the incorporation of labeled methionine into the muscle proteins of hypothyroid rabbits is compared to the incorporation by proteins of denervated muscles of control rabbits. As a rule, we observed in this case a decrease in the rate of incorporation of the labeled methionine into the muscle protein of the denervated extremity in comparison to that of the normal muscle; only in 3 cases did we observe an insignificant increase in the rate of protein regeneration in denervated muscle. The preliminary injection of 6-MTU exerts an even greater influence on the rate of oxidation. In this series of experiments the oxygen consumption of denervated muscle not only did not increase, but actually decreased in comparison to the oxygen consumption of normal muscle. Four experiments proved to be exceptions.

The activity of the enzyme pyrophosphatase increased significantly less than usually after muscle denervation in normal rabbits. In some cases, we could not observe any significant difference in the intensity of pyrophosphatase activity in normal and denervated muscles of hypothyroid animals, while in those rabbits which did not receive 6-MTU the activity of this enzyme increased up to 77-163% by the 19th to 21st day after the denervation.

DISCUSSION OF RESULTS

Denervation induces profound changes in the protein metabolism and oxidation processes of muscles.

TABLE 5

Protein Metabolism and Oxygen Absorption in Muscles of Hypothyroid Rabbits, after Denervation

Period of MTU injection prior to denervation (days)	Days after denervation	Muscle weight (g)			Proteolysis*			Methionine incorporation			Respiration (μl per 1 g)			Pyrophosphatase (μg of P)		
		Control	Denervated	Change (%)	Control	Denervated	Change (%)	Control	Denervated	Change (%)	Control	Denervated	Change (%)	Control	Denervated	Change (%)
60	13	4.2	4.2	0	78	83	+6.4	—	—	—	—	—	—	31	34	+9.7
75	14	3.6	3.2	—11.2	106	94	—11.4	—	—	—	—	—	—	44	43	0
75	18	3.9	3.6	—7.7	—	—	—	—	—	—	87.5	81.5	—6.9	15	16	+6.9
90	17	5.7	5.3	—7.0	—	—	—	—	—	—	176.5	150.0	—15.6	22	26	+18.2
90	19	3.0	2.8	—6.7	74	66	—10.8	—	—	—	68.0	62.0	—8.9	—	—	—
60	20	1.8	1.45	—19.5	140	156	+11.4	37	32	—13.5	81.0	100.0	+23.4	74	70	—5 4
57	17	1.52	1.1	—27.7	104	117	+12.5	68	78	+14.7	165.0	119.5	—27.6	56	59	+5.3
35	19	2.7	1.6	—40.8	277	231	—16.6	93	86	—7.6	141.0	127.5	—9.6	62	76	+22.6
27	19	2.8	1.7	—39.3	57	277	+386.0	81	127	+56	106.0	97.5	—8.1	—	—	—
26	19	4.1	2.8	—31.8	—	—	—	27	18	—33.4	132.0	100.0	—23.5	30	25	—16.7
30	19	2.4	2.0	—16.7	167	251	+50.3	42	40	—4.8	88.0	63.5	—24.5	33	42	+27.3
30	19	3.15	2.95	—6.4	151	223	+47.6	26	12	—53.9	118.0	95.0	—19.5	37	34	—8.0
40	19	2.35	1.9	—19.2	243	245	0	49	24	—51.0	185.0	186.0	0	40	50	+25.0
32	21	3.65	2.6	—28.8	80	210	+162.5	40	38	—5.0	77.5	74.0	—4.6	40	50	+25.0
17	21	2.32	1.36	—41.4	333	385	+15.6	50	47	—6.0	114.0	124.0	+10.6	60	61	+1.6
30	23	3.6	3.0	—16.7	125	142	+13.6	65	75	+15.3	145.5	110.0	—24.4	16	17	+6.2
40	25	3.5	2.3	—34.3	111	106	—4.5	67	37	—44.8	100.0	108.5	+7.5	31	35	+12.9
31	27	2.95	1.8	—39.0	97	161	+66.0	32	22	—31.3	82.0	99.5	+21.3	25	31	+24.0
31	28	4.7	2.6	—44.7	53	241	+354.7	29	27	—6.9	111.5	105.0	—5.9	60	80	+31.1

* Increase in protein-free nitrogen after incubation (mg %).

We succeeded in confirming the results obtained by other investigators on the increase of proteolysis, which apparently is mainly due to the degradation of functional proteins.

Changes in proteolytic activity may be observed soon after muscle denervation. This confirms the concept of the function of the nervous system in the structural organization of substances with high molecular weights.

The present work, as well as previous investigations [7] have shown that the intensity of protein metabolism in muscles of the denervated extremity is greater than that in the control. Preliminary experiments indicate an increase of the radioactivity of denervated muscles, regardless of the fact that the content of myosin and water-soluble proteins decreases under the same conditions.

The increase in amount and rate of protein synthesis lags behind the intensity of proteolysis. There is a possibility that the more intensive protein synthesis in denervated muscles is due to a greater permeability of the cellular membrane.

Somewhat similar observations on the rate of the synthesis of phosphorus compounds in muscle during atrophy due to vitamin E-free diet and to tenotomy were made by Ferdman [8] and Grigoryeva [9]. In spite of the fact that the amounts of adenosinetriphosphate and creatinephosphate were in these cases decreasing, the rate of their synthesis, determined by P^{32} incorporation, was significantly increasing.

The incorporation of amino acids is apparently coupled with the oxidation processes, which are intensified in denervated muscles. The respiration of denervated muscles was investigated by many authors in vivo and in vitro. At first glance the results obtained by various authors appear to be contradictory. Thus, in experiments on rats, a similar oxygen consumption was observed in normal and denervated muscles [10]. A decrease in respiration was observed in experiments on dogs [11] and toads [12], while an increase was observed in rabbits [13, 14]. It is possible that the various effects of denervation on tissue respiration in vitro are determined by the peculiar characteristics of the animal species.

In the majority of our experiments the respiration of denervated muscle was greater than that of normal muscle, which confirms the observations made by other authors on rabbits. If we take into consideration that the amount of water in denervated muscle increases, however little, and that the amount of muscle tissue decreases while the connective tissue increases, then the respiration of muscle tissue increases even more.

At the present time there is insufficient experimental evidence which could demonstrate what substances are oxidized and which oxidative systems are most active during the increase of the oxygen absorption in denervated muscle. It is possible that the activation of some oxidative enzyme occurs simultaneously with an increase of such substances as glutathione and ascorbic acid which are active in oxidative reactions in tissues. An indirect confirmation of this assumption may be found in the changes of the activity of pyrophosphatase. Kornberg established that pyrophosphate may form as a result of exchange reactions occurring during the synthesis of coenzymes (diphosphopyridine and flavin nucleotide) [15]. The pyrophosphatase is active in the removal of the pyrophosphate formed during this reaction. The relationship of pyrophosphatase with the oxidation reactions is indicated by the similar trend of the changes in respiration and of the activity of this enzyme in denervated muscles observed in experiments on normal and hypothyroid rabbits.

It was shown in experiments on animals receiving 6-MTU that inhibition of thyroid function substantially influences the degree of muscular atrophy and the intensity of oxidative processes.

A lesser degree of muscular atrophy was observed in hypothyroid animals after denervation than in rabbits not receiving 6-MTU. The muscular atrophy was significantly retarded in 6 rabbits which received 6-MTU for longer periods than others (60-90 days). The maximal loss of weight of the muscles of these animals, 13-20 days after denervation, was 19.5%, while during the same period the denervated muscles of normal rabbits lost 34.8-54.6%; no significant changes in proteolytic activity were observed in the same rabbits.

The intensity of methionine incorporation into the muscle proteins of hypothyroid animals was significantly decreased, and as a result the radioactivity of the proteins of denervated muscles was as a rule lower than normal.

The denervated muscles of rabbits receiving 6-MTU absorbed less oxygen than the muscles of the controls. In four experiments where an increase in the respiration of denervated muscles of hypothyroid animals was observed, the respiration was lower than in the corresponding muscles of the majority of control animals. The same trend is observed in the changes of pyrophosphatase activity.

The experiments which are being presently performed in our laboratory show that significantly smaller changes in the activity of glycolytic enzymes occur in denervated muscles of hypothyroid rabbits in comparison with those occurring in denervated muscles of normal rabbits. However, it must be mentioned that the changes in protein metabolism and oxidative processes of denervated muscles in rabbits receiving 6-MTU were the same as in normal rabbits.

In one case we obtained such a negative result with a pregnant rabbit. We also observed the absence of the influence of 6-MTU on the metabolism of denervated muscle in animals which significantly lost weight after denervation and which developed large trophic ulcers.

We observed the negative results most often during the spring, which may be related to the influence of sexual hormones on the action of 6-MTU. Voitkevich [16] observed the absence of the thyroid gland reaction to thiouracil in a number of animals, during the spring. He demonstrated that the introduction of methyl testosterone decreases the action of MTU.

We can thus state that the inhibition of the thyroid function significantly influences the protein metabolism and oxidative processes in denervated muscles. We did not observe any significant changes in the intensity of the above mentioned processes during the action of 6-MTU on normal muscles.

The above experiments confirm the assumption that denervation changes the hormone action in muscles. Kharvat [17] assumes that the basic property of the nervous system, in relation to hormones, is the protection that it offers to tissues against an excessively strong action of hormones. Apparently, the thyroid hormones exert an excessive influence on denervated muscles and a decrease in its amount will not prevent, but will noticeably inhibit, the development of disturbances which arise after denervation. The relation of the thyroid gland to denervated tissues is undoubtedly highly complex. It is impossible in the present work to give an idea of the intimate nature of the interaction between hormones and denervated muscles. However, the influence of the thyroid gland on the biochemical processes in denervated muscles presents an irrefutable interest and warrants further investigations.

SUMMARY

Muscle denervation leads to a more intensive incorporation of labeled methionine into protein molecules.

The intensity of respiration and the activity of the enzyme pyrophosphatase are increased in denervated muscles.

Atrophy, as a rule, is retarded after muscle denervation in hypothyroid rabbits. The proteolytic activity increases significantly less. In a majority of cases, no increase in radioactivity and oxygen absorption were observed in denervated muscles of these animals. The pyrophosphatase activity increases insignificantly.

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NATURE OF THE PRODUCTS OF DARK CO_2 FIXATION BY PLANT TISSUES

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After the discovery by Lebedev [1] of the assimilation of carbon dioxide by heterotrophic bacteria many investigations were performed indicating that the new type of carbon dioxide assimilation represented a problem of a general biological nature.

At the present time, it has been demonstrated that various groups of microorganisms, fungi and various plant and animal tissues are to some extent able to fix carbon dioxide in the dark, and that this reaction is essential to their life processes. However, the biochemical pathways of this assimilation are far from being completely explored.

Many substances which incorporate the carbon dioxide fixed in the dark and a number of enzymes catalyzing the formation of these substances are known. It has been shown, for instance, that some microorganisms are able to reduce this carbon dioxide to formic [2] and acetic acids [3]. Dark fixation and reduction of CO_2 by the hydrogenase of plant tissues has been discovered [4]. The widespread reactions of the carboxylation of keto acids, in particular of pyruvic [5] and α-ketoglutaric acids [6] with the formation of oxaloacetic and oxalosuccinic acids, have been studied. The formation of arginine has been shown to result from the addition of CO_2 and NH_3 to ornithine [7]. It has been recently shown that the carbon dioxide absorbed by bird liver enters into the composition of purine base [8].

Thus, the biochemical pathways of the dark carbon dioxide fixation are quite diversified.

There are two types of dark carbon dioxide fixation in plants. One type of absorption is directly related to photosynthesis and is expressed by its reactions occurring in darkness. This photosynthetic absorbtion of CO_2 quickly decreases in light and disappears after several minutes. The other, the dark fixation proper, takes place at a much slower but more constant rate. This fixation occurs in leaves and in other parts of the plant.

Since the investigations of Kursanov, Kryukova [9, 10] and their co-workers on the carbon dioxide nutrition of plant roots, which demonstrated a new and fast mechanism for the transportation and assimilation of carbon dioxide, and since the investigations of Kuzin and his co-workers [11], which demonstrated an intensive carbon dioxide assimilation from soil gases by isolated roots, it is undoubtedly true that the absorption of carbon dioxide by plant roots is an essential physiological process. In spite of great efforts, only the first steps have been taken toward the unraveling of the physiological significance and of the mechanism of the dark CO_2 fixation by plant leaves, and even so the existing information is somewhat contradictory. For instance, some investigators [12] conclude that the dark carbon dioxide fixation in green plants differs basically from that of heterotrophic fixation by type of β-carboxylation; other investigators [13] consider that CO_2 fixation occurs mainly, and perhaps exclusively, according to the reversible reaction of Wood-Werkman [5]. Most authors subscribe to their point of view; it is mainly based on the fact that many components and products of the di- and tricarboxylic acid cycles were identified during the dark CO_2 fixation by green plants. Benson and Calvin [14] identified malic, succinic, isocitric, glutamic and aspartic acids, alanine, serine and others.

While we admit such a mechanism of CO_2 incorporation into the above named compounds we cannot agree with the widespread belief that the dark carbon dioxide fixation is only a function of the concentration of CO_2 and that it is due mechanically to a simple reversibility of the respiration reaction. It must be remembered that

the biochemical processes often considered in general as simple reversible reactions are in fact complex, step-like processes determined at each step by a number of conditions. For this reason, the concentration of the initial and final substances cannot accurately reflect the trend of a process that is expressed, overall, as reversible reaction. The law of mass action is undoubtedly valid in living organisms, but its full value can be seen only in in individual reactions. For this reason, the direction of the reversible processes which are expressed overall by the chemical reactions of the dark carbon dioxide fixation cannot be explained by the chemical law of mass action alone. It is rather determined by a definite direction of the metabolism in conjunction with the physiological state of the organism. Casual and unnecessary biochemical reactions as well as reactions which are not more or less regulated by the organism are absent from the latter. The carbon dioxide completely surrounding the plant and constantly present in its tissues due to their respiration is used by the plant for photosynthesis and in dark metabolic reaction, and can at the same time be eliminated. We, therefore, consider that the dark fixation by the leaves and the carbon dioxide absorption by the roots are necessary physiological processes.

We have already pointed out the difference between the carbon dioxide absorption by green leaves resulting from dark reactions of photosynthesis and the dark absorption proper.

It has been previously demonstrated by Benson and Calvin [14] and by us [15] that the products formed in the dark after a short preliminary illumination of the plant differ little from the products formed under illumination. However, after the switching off of the light the relationship between the reactions definitely changes until an equilibrium characteristic of the processes occurring in plants growing in the dark, is reached. The amount of CO_2 absorbed and the nature of the products formed respond quickly to the disappearance of light. Our experiments resulted in the isolation of substances formed in the leaves of bean plants during 2, 10, 60 and 300 sec. upon dark CO_2 fixation proper, and after preliminary illumination. The experiments were performed according to the previously described method [15, 16] and with the application of radioactive carbon C^{14}.

Our radioautographs, obtained after long (up to 2 years) exposure of the film to the chromatogram show the difference in the initial directions of the two types of dark carbon dioxide fixation: that of the dark absorption proper, and that of the photosynthetic absorption, i.e., after preliminary illumination. The photosynthetic reaction seems to originate at one point, from one substance, identical to a previously isolated product under stationary photosynthetic conditions [17]. The same cannot be said about the direction of the dark absorption proper, during which no such initial point could be observed, regardless of the very short exposure. The carbon obtained during dark fixation appears on the chromatograms simultaneously from several points at the very beginning (Fig. 1A, B). This seems to indicate that there are several paths of simultaneous dark fixation of CO_2 in green leaves.

Upon lengthening of the exposure of the leaves to $C^{14}O_2$ the number of products formed is increased; it reaches 19 after 5 minutes. Organic acids (malic, succinic, fumaric, citric, α-ketoglutaric), amino acids (aspartic, glutamic, alanine) and amides of amino acids (asparogine and glutamine) were chromatographically identified among the products. The accumulation of these compounds linked with the di- and tricarboxylic acid cycles was discovered during 1 and 5 minute exposures of the leaves to $C^{14}O_2$. No accumulation was observed during the shortest exposures. Apparently, only certain intermediate compounds of the dark carbon dioxide fixation could be formed (in a somewhat different manner) during the first few seconds. There is still little known on the nature of these products, even those which are formed during the best known reactions. For instance, some authors [18, 19] assume that the incorporation of CO_2 into malic acid in animal tissues occurs with the aid of an unknown intermediate compound, rather than oxaloacetic acid as has been previously supposed. Phosphorus compounds have an important function in this reaction.

Numerous investigations of microbiologists, trying unsuccessfully to substitute completely the carbon dioxide which is essential for the normal development of heterotrophic bacteria by adding the final products of its dark fixation to the media mainly those products that are linked with the di- and tricarboxylic acid cycles, indicate that the function of carbon dioxide does not consist wholly of its participation in the replenishment of the stock of the above named compounds. Some investigators [20, 21] conclude on this basis that the function of carbon dioxide in heterotrophic organisms is still far from being ascertained. Apparently, the intermediate products formed during the various pathways of the dark carbon dioxide fixation and those reactions by which they are formed have a great significance in the life activities of animal and plant cells. The carbon dioxide often present in these reactions in infinitesimally small amounts, has essentially the action of a biochemical catalyst, contributing to the equilibrium of the reactions in the general metabolic processes.

Does CO_2 absorption by green leaves under light conditions occur by the mechanism of the dark reaction? The fact that we observed no products of dark CO_2 fixation among the products of photosynthesis on comparing

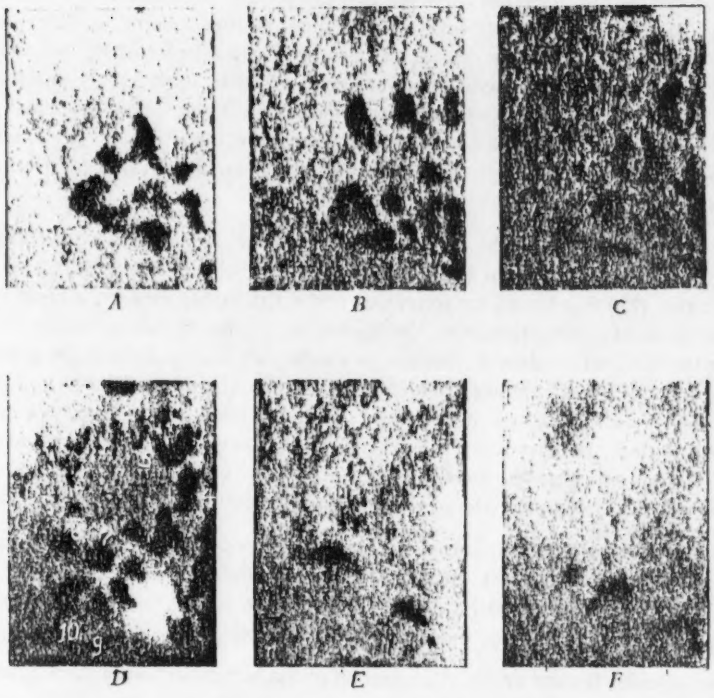

Fig. 1. Radioautographs of chromatograms. Solvents: horizontally – phenol saturated with water; vertically – butyl alcohol-acetic acid-water (74:20:50). 1) Malic acid; 2) succinic acid; 3) fumaric acid; 4) citric (or isocitric ?) acid; 5) α-ketoglutaric acid; 6) aspartic acid; 7) glutamic acid; 8) alanine; 9) asparagine; 10) glutamine. A) darkness, 2 sec.; B) darkness, 10 sec.; C) darkness, 60 sec.; D) darkness, 300 sec.; E) stationary photosynthesis, 1 sec.; F) preliminary illumination followed by darkness, 1-2 sec.

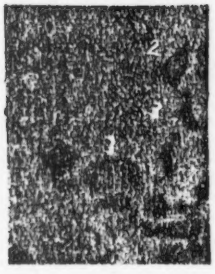

Fig. 2. Darkness (5 minutes) followed by illumination (40 seconds).
1) Initial product of photosynthesis; 2) malic acid; 3) citric acid.

Fig. 3. Repeated chromatogram of the eluted initial product of the photosynthesis, formed: 1) after dark CO_2 adsorption; 2) during stationary photosynthesis.

the chromatograms obtained during experiments with 1-2 seconds exposures of the leaves in the presence of labeled CO_2 in light and darkness, is not sufficient evidence that carbon dioxide fixation does not occur according to the mechanism of the dark reaction during illumination. It must be remembered that the rate of photosynthesis in our experiments was higher than the rate of dark CO_2 absorption by a factor of 10, 100 and even 1000. For this reason the carbon dioxide entering the leaves in normal concentration is practically wholly used in photosynthesis. It is possible that under these conditions the carbon dioxide eliminated by the tissues during their respiration will be more accessible to the dark absorption, however, it will not contain any labeled carbon dioxide, and, moreover, it will be used in photosynthesis to a large extent, thus making its detection in the products of dark CO_2 fixation impossible.

Since we were primarily interested in the least investigated elementary and initial reactions of dark fixation, we were compelled to devote most of our attention to the initial point of this fixation, i.e., the initial CO_2 adsorption. We demonstrated [22] that before the formation of the first stable product, a rapid and unstable bonding, probably due to adsorption of carbon dioxide by the leaves, occurs during photosynthesis. A similar phenomenon occurs also during the dark carbon dioxide fixation in leaves. We took samples from halves of the same leaves kept in the dark. After 1-2 sec. exposures in a dark room containing 0.1-0.2% $C^{14}O_2$ in the air a part of the samples was immediately fixed by hot steam and dried, while the other, the control, was immediately illuminated and fixed only after 5 min. In other series of experiments the length of the exposures to darkness and light were varied. Measurements of the radioactivity of the samples showed that the carbon dioxide is adsorbed by the leaves in darkness and then is fixed by photosynthesis during illumination (Table 1).

It is also apparent from Table 1 that the initial amount of adsorbed carbon dioxide is eliminated from the leaves if they are subsequently kept in the dark for several minutes. However, under other dynamic conditions the carbon dioxide absorbed at the outset in large concentrations in the dark [23] may be retained by the leaves for a long time and be subsequently used in the process of photosynthesis.

Examination of the products formed during illumination of the leaves following their adsorption of carbon dioxide in the dark demonstrates convincingly that they do not as a whole differ from the usual products of photosynthesis (Table 2).

Among these products we identified a substance isolated by us previously [17] which under normal conditions of photosynthesis is formed as the initial stable product. In certain experiments we chose conditions of exposure to darkness and light under which this substance would appreciably predominate among the other products of photosynthesis and of dark CO_2 absorption.

TABLE 1

Unstable Bonding of $C^{14}O_2$ by the Leaves in Darkness, and Its Subsequent Assimilation During Photosynthesis

Expt. No.	Conditions under which the leaves were exposed to $C^{14}O_2$	Duration of exposure (sec.)	Conditions after exposure	Radioactivity (cpm per cutting)
1	Illumination	1-2	Killed	931
2	Illumination	1-2	Killed	1024
3	Illumination	1-2	Killed	1764
4	Darkness	1-2	Killed	34
4	Darkness	1-2	Illumination, 5 min.	581
5	Darkness	1-2	Killed	52
5	Darkness	1-2	Illumination, 5 min.	737
6	Darkness	1-2	Killed	25
6	Darkness	1-2	Illumination, 5 min.	685
7	Darkness	60	Killed	917
7	Darkness	60	Illumination, 5 min.	47550
8	Darkness	1-2	Killed	27
8	Darkness	1-2	Darkness for 5 min. followed by illumination for 5 minutes.	61

TABLE 2

Radioactivity Distribution Among Fractions After $C^{14}O_2$ Fixation Under Various Conditions

No.	Exposure conditions in room containing $C^{14}O_2$	Conditions in the room, after exposure	Radioactivity in fractions of kidney bean leaves (%)				
			insoluble in water	insoluble in 80% alcohol	of anions	of cations	of neutral substances
1	Illumination, 1 min.	Killed	9.6	50.3	17.1	10.6	13.0
2	Darkness, 1 min.	Killed	0.8	38.6	54.5	5.8	0.3
3	Illumination, 2 sec.	Illumination, 5 min.	29.5	27.2	15.3	10.6	17.4
4	Darkness, 2 sec.	Illumination, 5 min.	23.6	31.3	18.8	11.2	15.1

This substance was very distinct on the chromatograms obtained in separate experiments, giving an intensive darkening of the x-ray film (Fig. 2). After being eluted and rechromatographed this substance proved to be identical with the previously isolated initial product of photosynthesis (Fig. 3). It follows that the initial adsorption of CO_2 in light and darkness is of the same nature. In order to obtain some information on the physiological aspects of this problem we investigated the dark CO_2 absorption in various plant tissues. One sample of the material was killed by steam at 100° after a 1 minute exposure in the presence of $C^{14}O_2$, and another identical sample was killed after being kept for 12 hours in a dark humid room. The results of these experiments are given in Table 3.

TABLE 3

Dark Absorption and Decarboxylation of $C^{14}O_2$ in Various Plant Tissues.

No.	Material	Conditions of $C^{14}O_2$ absorption	Radioactivity (cpm)		Radioactivity (%)	
			Immediately after 1 min. exposure	After 12 hours in darkness	Immediately after 1 min. exposure	After 12 hours in darkness
1	16 day old kidney beans, leaves	Illumination	171244	113030	100	66.1
2	16 day old kidney beans, leaves	Darkness	6324	2202	100	39.7
3	10 day old kidney beans, leaves	Darkness	9105	5480	100	60.2
4	10 day old kidney beans, etiolated leaves	Darkness	4156	1759	100	42.2
5	Kidney beans, sprouts	Darkness	1052	715	100	68.0
6	Kidney beans, roots	Darkness	354	102	100	31.8
7	Primrose, leaves	Darkness	815	344	100	42.3
8	Primrose, roots	Darkness	132		100	
9	Primrose, petals	Darkness	160	19	100	11.8
10	Poppy, petals in blossom	Darkness	863	125	100	14.5
11	Poppy, falling petals	Darkness	104	4	100	3.9

From the above data it is apparent that various plant tissues differ sharply not only in the intensity of their dark carbon dioxide absorption, but also in their subsequent utilization of it. The great heterogeneity of the material prevented the comparative studies from being carried far enough to provide a conclusive explanation of the facts. However, the available information is of definite interest and permits us to draw certain conclusions. The youngest tissues are distinguished by the greatest intensity of CO_2 absorption. At the same time the C^{14} absorbed by these tissues is apparently found in compounds with which it becomes further involved in metabolic processes and its removal by decarboxylation is thereby retarded.

SUMMARY

Upon studying, by means of labeled atoms and chromatography, the dynamics of the products of the dark CO_2 absorption in leaves of Phaseolus a number of substances linked with the di- and tricarboxylic acid cycles were found. Among them, organic acids (malic, succinic, fumaric, citric, α-ketoglutaric), amino acids (aspartic, glutamic, alanine), amides of amino acids (asparagine, glutamine), and a number of other unidentified substances were present.

The existence of several independent pathways of the dark CO_2 absorption in leaves was determined.

The existence of a common labile precursor of the light and dark CO_2 fixation in green leaves was demonstrated.

Some characteristics of dark CO_2 absorption in various plant tissues were observed.

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ENZYMATIC DEGRADATION OF DEXTRAN BY SPLEEN EXTRACTS

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In spite of the fact that polyglycosides of the dextran type have been known for a long time, the process of their enzymatic degradation has been studied insufficiently to the present time.

Dextrans of various origins differ to a significant extent in their chemical structure. A branched structure is characteristic for a majority of them. Side chains of various lengths branch off from glucopyranose chains in which the glucose residues are linked by 1, 6-glycosidic bonds. Cross-linkages in molecules of various dextrans occur in the 1, 3- or 1, 4- positions. Dextrans are synthesized by various microorganisms. The properties of "dextranases" extracted from cultures of these microorganisms and the mechanism of their action are not clear to the present time.

In spite of the fact that 1, 6-glycosidic bonds always predominate in dextran molecules, the presence of other bonds (1, 3- and 1, 4-) forces us to assume that at least 2 or 3 enzymes participate in the processes of synthesis and degradation of dextrans.

The process of enzymatic degradation of dextrans has been studied much less than the process of their synthesis. Dextrans are not split by amylases (salivary, malt, soya β-amylase and takadiastase [1]), muscle and potato phosphorylases in the presence of phosphates [2], or by the isomerase of amylase [3] which splits 1, 6 -glycosidic bonds at the branching points of starch and glycogen polysaccharides.

The degradation as well as the synthesis of dextrans has been investigated up to now only with the aid of enzymes of bacterial origin. The presence of dextran-splitting enzymes in animal organisms has not yet been demonstrated. However, facts are now being accumulated that testify to the probability of an enzymatic breakdown of dextrans in the organism. C^{14} has been detected in the carbon dioxide exhaled [4] and in the glucose excreted in the urine [5] following the introduction of dextran labeled with C^{14} into man and animals.

The possibility of the degradation of dextran in the organism is important in connection with its use as a substitute for blood plasma. For this reason numerous investigations have been devoted in recent years to searches of enzymatic mechanisms able to accomplish their degradation. However, no one has yet succeeded in detecting dextran-splitting enzymes in various tissues and organs of animals [6-8].

We [9] demonstrated that the enzymatic degradation of dextrans may occur with the aid of spleen extracts or homogenates. In the present paper we report the data concerning the characteristics of the splitting of dextran by enzymatic preparations obtained from spleen extracts.

The enzymatic preparations that split dextran were prepared in the following manner. The spleens were carefully cut into small pieces and extracted for 2-3 hours by equal weights of 0.2 M acetate buffer, pH 4.8. The extract obtained after centrifugation in a number of experiments was autolyzed (for the degradation of the polysaccharides present in spleen extracts) and dialyzed (for the removal of reducing substances). A part

of the proteins present in the extract was removed by centrifugation after usual precipitation during dialysis. Purification of the obtained spleen extracts was subsequently carried out by fractional precipitation with $(NH_4)_2SO_4$. $(NH_4)_2SO_4$ was added to the solution to 0.3 saturation. The residue formed after centrifugation was rejected and the solution saturated with $(NH_4)_2SO_4$ to 0.4-0.5 saturation. The obtained precipitate was dissolved in water and dialyzed for 24 hours against tap water and then distilled water. The preparations were further purified by 2 or 3 reprecipitations with $(NH_4)_2SO_4$, 0.4-0.5 saturated. The activity of such preparations was significantly higher than the activity of the initial extracts. The results obtained with the purified preparations of enzymes are shown on the figure.

The optimum hydrogen-ion concentration for the splitting action of the spleen extract is, as shown by us previously, equal to 4.8. At pH 6.0 the activity of the extract is significantly lowered, and at a higher pH the extracts do not split dextran at all.

The extent of degradation of the dextrans was estimated by the increase in reduction during their incubation with extracts. The reduction of the incubated mixture (dextran + extract + buffer) was determined before incubation. The reduction of the control (extract + buffer) was subtracted from the obtained results. The reduction after incubation was determined in the same manner. The reduction of the control autolyzed and dialyzed preparations and of the preparations obtained after ammonium sulfate fractionation was usually nil before as well as after incubation.

Upon incubation of dextran with extracts of rabbit spleens for 24 hours, the accumulation of reducing substances in the incubating mixture corresponded in various experiments to a 5-15% degradation of the dextran under optimum conditions. As mentioned previously, the purified preparations are characterized by a significantly higher activity, and the accumulation of reducing substances, upon the degradation of dextran for the same time of incubation, is greater (approximately 25%).

The degradation of dextran could be detected not only by the accumulation of reducing substances, but also by its decrease, in the incubating mixture. Therefore, the proteins were separated by trichloroacetic acid in a part of the samples, before and after incubation; the dextran was precipitated and again reprecipitated by alcohol from an aqueous solution, dried to a constant weight, and weighed.

The content of dextran was simultaneously determined in boiled extracts, before and after incubation. An insignificant, but always definite, decrease in weight, equal to approximately 5-8% of the initial weight of dextran, was observed in experiments with fresh, but not boiled, extracts (Table 1).

TABLE 1

Degradation of Dextran By Spleen Extract

Experiment No.	Extract	Weight of dextran (mg)	
		Before incubation	After incubation
1	Fresh	88.4	80.1
	Boiled	89.1	88.1
2	Fresh	66.7	62.1
	Boiled	67.1	67.3
3	Fresh	66.3	62.0
	Boiled	65.8	66.0

In order to eliminate in our experiments the possibility of the degradation of dextran by microorganisms, we incubated dextran with spleen extracts under sterile conditions.

The incubating mixture was filtered through a Seitz filter. Bacterial growth was checked (by inoculating sugar bouillon, agar, blood plasma, etc.) before, and 2-3 days after, the beginning of incubation. No bacterial growth could be detected under these conditions. An analogous experiment was simultaneously performed under non-sterile conditions.

As seen from Table 2, the degradation of dextran under sterile and non-sterile conditions was the same.

TABLE 2

Extent of Dextran Degradation By Sterile Extracts From Spleen

Experiment No.	Conditions of experiment	Degradation of dextran	
		(mg/ml)	(%)
1	Sterile	0.324	4.5
	Non-sterile	0.360	5.0
2	Sterile	0.552	7.7
	Non-sterile	0.480	6.7

Spleen extracts of various animals such as dogs, rabbits, rats and oxen have the ability to split dextran. The activity of the obtained enzymatic preparations varied approximately within the same range regardless of the animal species from which it had been taken. However, extracts from ox spleen usually were somewhat more active (Table 3).

TABLE 3

Extent of Dextran Degradation By Spleen Extracts of Various Animals

Incubation mixture: 20 mg of dextran, 1.0 ml of 0.2 M acetate buffer (pH 4.8), 1.5 ml of extract; incubated for 48 hours.

Animal	Number of experiments	Degradation of dextran (%)
Dog	6	5-13
Rabbit	12	4-15
Rat	1*	7
Ox	23	8-25

* 15 rats were used in this experiment

The greatest extent of dextran degradation was detected 48-72 hours after the beginning of incubation, and in the case of active purified and dialyzed preparations it amounted to 25-30% of the weight of the initial dextran. Upon further incubation there was practically no further degradation of the dextran.

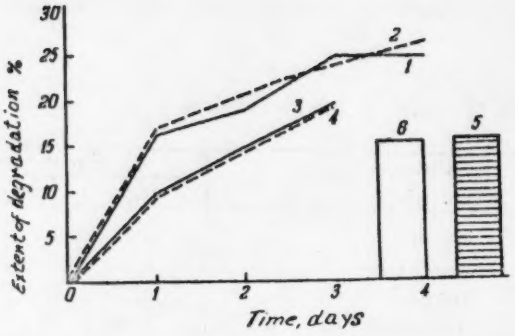
The activity of purified enzymatic preparations did not increase in the presence of NaCl or Mg ions, and was not inhibited by sodium fluoride (see Fig. 1).

Upon incubation of dextran with the enzymatic preparations in the presence of phosphates no decrease in the weight of inorganic phosphate was observed. This testified to the fact that the degradation of dextran did not occur by phosphorolysis. The extent of the degradation of dextran depends on the activity of the enzyme preparations as well as on the structure of the various dextrans.

We used the following "clinical", i.e., the partly depolymerized, preparations of dextrans: the domestic polyglucosans * (soluble and "insoluble") as well as the English and Swedish preparations "Intradex" and "Macrodex".

It was found that upon the same period of incubation under the same conditions and during the simultaneous action of the same extract on various dextrans, the extent of degradation of the latter varies.

* The polyglucosans, obtained from partially hydrolyzed, soluble or insoluble dextran, were prepared at the Central Institute of Hematology and Blood Transfusion.



Extent of dextran degradation by purified preparations of spleen extract: 1) NaCl; 2) without NaCl; 3) NaF; 4) without NaF; 5) $MgCl_2$; 6) without $MgCl_2$.

As seen from Table 4, the degradation is approximately the same for Macrodex and polyglucosans (insoluble and soluble, 1) – 15-16%, somewhat higher for Intradex – 18.7%, and significantly lower for the soluble polyglucosan, 2 – 6.1%.

We performed the following experiment in order to determine the reason for the variable degradation of different dextrans: the dextran preparation was submitted to the action of the extract for 24 hours, after which the proteins were precipitated in a portion of the incubating mixture by the addition of trichloroacetic acid and a part of the dextran ("residual dextran" I) was eliminated from the solution. A fresh portion of extract was added to the remaining incubating mixture and after incubation for 24 hours the ("residual dextran" II) was precipitated and separated from the solution. The preparation III was separated in the same way after 24 hours. All the obtained preparations were purified, dried and then submitted simultaneously to the action of the same extract. The content of dextran in each sample was determined by acid hydrolysis. The results obtained are given in Table 5.

TABLE 4

Extent of Degradation by Spleen Extracts of Various Preparations of Dextran

Preparations	Extent of degradation (%)
Polyglucosan	
"Insoluble"	15.9
Soluble 1	15.0
Soluble 2	6.1
Intradex	18.7
Macrodex	15.0

TABLE 5

Extent of Degradation of "Residual" Dextrans By Spleen Extracts (Incubation: 48 hours)

Dextran Preparation	Extent of degradation	
	(mg/ml)	(%), on the basis of the degradation of the initial preparation
Initial	1.08	100
"Residual" I	0.52	48.5
"Residual" II	0.30	27.5
"Residual" III	0.10	8.8

Thus, the longer the dextran preparation is submitted to a preliminary action of enzymes, the smaller will be its degradation by the given enzyme. This fact leads us to the assumption that dextran molecules have bonds (possibly links between branching chains) that are inaccessible to the action of the enzyme present in spleen extracts. Upon partial degradation of dextran molecules the number of bonds the splitting of which is catalyzed

by the given enzyme preparation, decreases. If so, the variations which occur in the extent of degradation of various dextran preparations are most probably related to the length of the terminal branches of their molecules.

DISCUSSION

The obtained results testify to the fact that a dextran-splitting enzyme is present in spleen extracts. Only 20-25% of the dextran is degraded probably owing to the presence in the dextran molecule of bonds that are inaccessible to the action of this enzyme.

It may be interesting to correlate our assumption that the partial degradation of dextran leads to a shortening of the terminal branches of its molecules with the fact that the dextrans of low degradability (the "soluble" polyglucosans 2), in contrast to the dextrans with a relatively high degradability, do not combine with proteins. This is in accordance with the results [10] which indicated that the length of the outside branches of the molecules of branched polysaccharides is important in the process of their association with proteins since shortening of the terminal branches of polysaccharide molecules leads to a loss of their ability to form complexes. Apparently the same conformity is observed in dextrans.

Various pathways of dextran degradation by enzymes of microorganisms have been described in the literature. Ingelman [11] describes the degradation of dextran under the influence of Cellvibrio fulva characterized by an insignificant increase in reduction (3% of the weight of dextran after 24 hours of incubation) and a significant decrease in the viscosity of the preparation which, according to him, testifies to the splitting of the dextran molecule into large molecular fragments. Jeanes and his co-workers [12] describe the degradation of dextran under the influence of enzymes from Penicillium funiculosum which occurs with a rapid accumulation of reduction (77% degradation of the dextran after 4 hours and 94% degradation after 5 hours of incubation, and the formation of mono-, di- and trisaccharides: glucoses, isomaltoses and isomaltotrioses).

The fact that upon the action of spleen enzymes, high molecular dextrins are formed, while the decrease in the viscosity of the preparations is insignificant, provides a basis for the assumption that the character of the denaturation of dextran is in our experiments different from that in the experiments of Jeanes and Ingelman.

SUMMARY

Spleen extracts from various animals (ox, rabbit, dog, rat) have the ability to split dextran. Upon fractionation of the extracts from ox spleen an active enzyme fraction was extracted by 0.4-0.5 saturation with ammonium sulfate.

Preparations of various dextrans are characterized by various extents of degradation by spleen extracts. After a preliminary action of the extract on dextran, the extent of degradation of the obtained "residual dextran" is sharply decreased.

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ENZYMATIC DECOMPOSITION OF L-HISTIDINE IN LIVER AND KIDNEYS OF NORMAL AND TUMOR-AFFECTED ANIMALS

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As shown by previous experiments performed in our laboratory [1, 2] the breakdown of a molecule of histidine begins in the liver by the process of deamination under the action of a specific deaminase. The urocanic acid formed by this reaction is converted by urocaninase, a second enzyme found in the liver, into a new compound, the structure of which, while not being yet ascertained, is certainly not that of glutamic acid [3]. This product of the conversion of urocanic acid is in turn enzymatically split further in the kidneys.

In the present work, we proposed to follow in greater detail the excretion in urine of the product of urocanic acid conversion in various animals and man after the administration of fairly large quantities of histidine and the enzymatic catabolism of the end-products of the conversion of histidine in kidneys.

At the same time the deamination of the α-amino group and the breaking of the imidazole ring of histidine in the liver occurring without the formation of glutamic acid, compelled us to investigate the problem of the function of hydrogen in these processes. Such experiments became especially desirable after the discovery of the activating action of histidine on the oxidase of d-amino acids [4]. Finally, the disruption of the metabolism of histidine upon development of malignant tumors [2, 5, 6] was the reason for undertaking analogous investigations in the present work.

Experiments on Animals and Man

It has been demonstrated in a previous report that the product of urocanic acid conversion forming in the liver is not excreted in the urine [2] even after subcutaneous injections of histidine to normal and tumor-affected rats. Due to the important significance of these experiments in ascertaining the mechanism of enzymatic conversion of histidine in animal organisms we repeated them on rats and humans as well as on other normal and tumor-affected animals.

The experimental animals were kept in suitable cages for the collection of the 24 hr. urine. The weight of experimental animals was: 118-224 g for rats, 1850-2050 g for rabbits, 13-15 kg for dogs. The humans under observation weighed 55-70 kg. The experimental animals received a characteristic diet for each specie. Only healthy rats were switched to a carrot diet two days before the administration of histidine. This diet was maintained for one day after the administration. Humans received a total diet. The following determinations were made in the urine: total nitrogen, urea – by the urease method [7], amino nitrogen – by the copper method [8, 9], ammonia – according to Folin with the displacement of the diazo compound by sodium carbonate, and sodium hydroxide by the diazo test modified by Meshkova. As tumor-affected animals we used rats with rhabdomyoblastoma (MOP) and rabbits with the Brown-Pearce tumor. Some experiments were performed on females with cancer of the mammary gland. Histidine was injected subcutaneously into animals after neutralization of its hydrochloric acid by sodium hydroxide. In experiments on humans the same preparation was administered either subcutaneously or per os.

It is apparent from Table 1 that in all experimental animals and humans the content of ammonia in urine (displacement with sodium carbonate and sodium hydroxide) remains constant in spite of the large amount of administered histidine. This indicates the absence in the urine of the product of urocanic acid conversion, since in its presence more ammonia should have been displaced by sodium hydroxide than by sodium carbonate.

Thus, our previous observations on the enzymatic degradation in the animal organism of the product of urocanic acid conversion which forms in the liver were again confirmed.

TABLE 1

Excretion in the Urine of the Final Products of Nitrogen Metabolism Before and After Histidine Administration (g)

Animals and humans	Amount of administered histidine	Total nitrogen	Urea nitrogen	N — NH_3		N — NH_2	Diazo compound
				Na_2CO_3	NaOH		
Healthy rat	0	0.086	0.054	0.006	0.007	0.001	0.001
	1.0	0.293	0.108	0.015	0.020	0.063	0.609
" "	0	0.078	0.043	0.005	0.005	0.001	0.005
	1.0	0.183	0.040	0.019	0.021	0.028	0.300
Rat with MOP tumor	0	0.099	0.081	0.010	0.010	0.002	0.001
	0.5	0.316	0.225	0.030	0.034	0.039	0.230
Same	0	0.148	0.114	0.013	0.014	0.002	0.006
	0.5	0.275	0.121	0.026	0.027	0.041	0.237
"	0	0.149	0.069	0.006	0.008	0.002	0.016
	0.5	0.249	0.125	0.015	0.016	0.030	0.159
"	0	0.108	0.094	0.008	0.009	0.002	0.001
	0.5	0.235	0.145	0.022	0.022	0.024	0.168
Healthy rabbit	0	0.987	0.467	0.027	0.031	0.026	0.017
	1.5	1.141	0.645	0.027	0.036	0.099	0.429
" "	0	0.667	0.482	0.006	0.007	0.037	0.017
	1.5	1.128	0.542	0.020	0.029	0.214	0.714
Rabbit with Brown-Pearce tumor	0	0.403	0.323	0.006	0.006	0.015	0.015
	1.0	0.401	0.245	0.005	0.006	0.011	0.058
Same	0	0.376	0.268	0.015	0.015	0.013	0.015
	1.0	0.508	0.229	0.014	0.014	0.027	0.086
Healthy dog	0	5.030	2.639	0.319	0.266	0.064	0.068
	6	6.769	5.428	0.541	0.585	0.163	0.112
" "	0	5.871	4.536	0.528	0.562	0.098	0.095
	10	7.050	5.430	0.519	0.584	0.242	0.277
Healthy woman	0	11.820	9.490	0.570	0.650	0.260	0.260
	6	11.360	7.890	0.880	0.880	0.337	0.445
Woman with cancer of the mammary gland	0	7.640	6.540	0.340	0.340	0.082	0.079
	6.0	8.680	7.180	0.310	0.310	0.073	0.102
Same	0	6.260	4.140	0.650	0.700	0.180	0.226
	10.0	7.560	4.980	0.660	0.730	0.206	0.450
	0	7.440	5.570	0.280	0.298	0.109	0.110
"	10.0	7.630	5.720	0.347	0.395	0.105	0.215

Degradation of Histidine in the Liver

For a long time it has been thought that histidine degradation in the liver was catalyzed by the enzyme histidase under anaerobic conditions. However, according to our data, there is no separate and independent histidase enzyme present in the liver. In fact the breakdown of histidine in this organ is catalyzed by two enzymes: histidine deaminase and urocanase. This fact, in addition to the profound changes that the molecule of histidine undergoes in the liver — expressed by the removal of the α-amino group in the form of ammonia, the formation of urocanic acid, and the breaking of the imidazole ring of the latter — have naturally led us to the investigation of the function of hydrogen in these reactions. Therefore, we performed experiments with liver homogenates of normal and tumor-affected rats, in the Warburg apparatus under aerobic and anaerobic conditions (in an atmosphere of nitrogen).

1.1 ml of Ringer-Krebs solution, pH 7.4-8.4, and 400 mg of liver homogenate introduced into the main vessel of the Warburg apparatus were used in each experiment. 1 ml of Ringer-Krebs solution containing 4 mg

TABLE 1

Breakdown of Histidine in Homogenates and Extracts of Liver (Ammonia, Amino Nitrogen and Histidine, in mg)

Enzyme source	Administered histidine	N – NH_3		N – NH_3				O_2 Absorption (QO_2)				Breakdown of histidine (%)
		Na_2CO_3	NaOH	Na_2CO_3 5 min	Na_2CO_3 10 min	NaOH 5 min	NaOH 10 min	Control	Experimental	Difference	Duration of Exp't., hrs.	
In an atmosphere of O_2												
Liver homogenate of healthy rat								2.99	3.99	1.0	1	
								1.68	1.36	—0.32	1	
Same	5	0.25	0.43					4.67	5.35	0.68	2	48
								4.59	4.78	0.19	1	
								3.45	3.61	0.16	1	
Same	4	0.15	0.30					8.04	8.39	0.35	2	41
								2.59	3.27	0.68	1	
								1.71	2.15	0.44	1	
	4	0.18	0.34					4.30	5.42	1.12	2	47
Liver extract of healthy rat	10	0.56	1.61								10	89
	10	0.64	1.62								16	90
Liver extract of cat	20	1.82	3.57	0	0	1,43	1,93				24	100
	20	1.87	3.48	0	0	1,36	1,74				24	96
	20	1.68	3.54	0	0	1,17	1,77				24	98
Liver homogenate of tumorous rat (MOP)								3.13	3.23	0.10	1	
								1.97	2.12	0.15	1	
Same	4	0.13	0.21					5.10	5.35	0.25	2	30
								4.09	4.47	0.38	1	
								2.80	2.50	—0.30	1	
Same	4	0.11	0.18					6.89	6.97	0.08	2	25
								2.55	2.68	0.13	1	
								1.50	1.57	0.07	1	
Same	4	0.10	0.15					4.05	4.25	0.20	2	20
In an atmosphere of N_2												
Liver extract of healthy rat	10	0.56	1.34								10	74
	10	0.64	1.58								16	87
Liver extract of cat	20	1.05	3.15								12	87

of histidine was introduced into the side-bulb. In control experiments the side-bulb contained 1 ml of Ringer-Krebs solution without histidine. At the end of each experiment, which lasted usually for 2-4 hours, the proteins were precipitated by 1 ml of 14% trichloroacetic acid. The following were determined in the filtrate: nitrogen, according to Folin by displacement with sodium carbonate and sodium hydroxide; histidine and

urocanic acid, by the diazo reaction as modified by Meshkova. In addition to the experiments in the Warburg apparatus we performed macro experiments with homogenates and extracts of rat and cat liver under aerobic and anaerobic conditions (nitrogen atmosphere) in which 10-20 mg of histidine was used. The techniques of such experiments are described in detail in our previous work [2].

These experiments were not performed exclusively to investigate the influence of the presence of hydrogen on the enzymatic degradation of histidine in the liver. We also hoped to obtain by these experiments new information on the mechanism of the biological degradation of histidine and on the nature of the intermediate compound formed upon alkaline hydrolysis of the product of urocanic acid conversion. Contrary to the numerous published data, we did not confirm the formation of glutamic acid or its derivatives in the form of intermediate or end-products of the degrading molecule of histidine, although we used the most precise methods of identification, such as the preparation of its hydrochlorides, precipitation in the form of barium salts, qualitative and quantitative paper chromatography [2], and the separation of glutamic acid upon degradation of large amounts of histidine by cat liver extracts [3].

Our determinations of amino nitrogen also deserve special attention. If glutamic acid or any other α-amino compound were present, all the amino nitrogen should have completely reacted with the nitrous acid upon 5 minutes agitation in a Van Slyke apparatus. However, as reported by us previously [2], the amount of amino nitrogen determined under the above conditions in the compound formed by alkaline hydrolysis of the product of urocanic acid conversion is always smaller (by an average of approximately 25%) than the theoretically calculated amount. This observation did not surprise us, since it is in full agreement with the experimental data and the theoretical considerations. In fact, the enzymatic splitting of the α-amino nitrogen from histidine in the form of ammonia and the formation of urocanic acid are strong indications that no α-amino compound will be found among the products of histidine degradation. There is no doubt that the amino group of the yet unknown substance forms only upon disruption of the imidazole ring of urocanic acid under the action of the enzyme urocanase and subsequent treatment of the product of the latter reaction by strong alkali (30% NaOH). It is difficult to conceive of the formation of an α-amino group upon breaking of the imidazole ring. A. N. Parshin has suggested that the formation of a γ- or possibly δ-amino compound is most probable. In order to obtain some experimental evidence for the solution of the problem under consideration, we performed in the present work comparative determinations of the amount of amino nitrogen in the Van Slyke apparatus upon agitation for 5 and 10 minutes. The results of a part of our experiments concerning the breakdown of histidine in the liver are presented in Table 2.

Like other authors [4, 10-12], we observed no increase in oxygen absorption during the degradation of histidine by liver extracts. It is true that in individual cases we sometimes observed a greater utilization of oxygen by liver extracts in the presence of histidine during the first hour of the experiment. However, this increase in respiration is not connected with the enzymatic conversion of histidine, inasmuch as the breakdown of the given amino acid in homogenates and extracts of rat and cat liver is absolutely identical under aerobic as well as anaerobic conditions.

Interesting results were obtained during the investigation of amino nitrogen. It was found that all the theoretically calculated amino nitrogen of the substance forming upon alkaline hydrolysis of the product of the enzymatic conversion of urocanic acid reacts completely with nitrous acid only upon agitation in the Van-Slyke apparatus for 10 min. We interpret these observations as a substantial confirmation of our hypothesis of the possible formation of a γ- or δ-amino acid upon alkaline hydrolysis of the product of urocanic acid conversion, inasmuch as the reaction of the α- and even β-amino group with nitrous acid in the Van-Slyke apparatus is usually completed within 5 minutes.

It goes without saying that more detailed investigations, even the isolation of the new unknown amino compound in the pure state, are necessary for the final solution of such a fundamental problem. In conclusion we should call attention to the decrease in the intensity of histidine degradation in the liver of tumor-affected rats in comparison with the degradation in the liver of healthy animals.

Degradation of the Product of Urocanic Acid Conversion in Kidneys

We have seen that the enzymatic conversion of a molecule of histidine in the liver terminates at the stage of the degradation of urocanic acid by the enzyme urocanase. At the same time, we could not detect the product of the given reaction in the urine of animals or humans even after the subcutaneous injection of large amounts of histidine. Therefore, it became clear that other organs were active in the metabolism of histidine in animal organisms. We subsequently detected in the kidneys a new enzyme system which, similarly to alkali,

breaks down the product of urocanic acid conversion with the formation of an amino compound. The experiments concerned with this part of our investigations were performed in several ways on homogenates and extracts of cat liver and kidneys exclusively, since the enzymes catalyzing the breakdown of histidine in these animals are characterized by maximum activity.

TABLE 3

Breakdown of Histidine in Homogenates and Extracts of Cat Liver and Kidneys (Ammonium, Amino Nitrogen and Histidine, in mg)

Enzyme source		N – NH_3		N – NH_3				O_2 Absorption (QO_2)			Duration of Expt., hrs.
				Na_2CO_3		NaOH					
		Na_2CO_3	NaOH	5 min.	10 min	5 min	10 min	Control	Experiment	Difference	
				In an atmosphere of O_2							
	20	2.84	4.30	0.81		1.44					24
	20	2.33	3.31	0.99		1.27					24
	20	2.83	3.56	1.29		2.64					24
Liver extract + kidney homogenate	20	2.33	3.80	0.94	2.35	1.58	2.65				24
	20	2.02	3.30	1.17	1.86	1.17	1.92				24
	20			1.44	1.73	1.55	2.01				24
	20			1.20	1.73	1.44	2.01				24
											24
	20	2.32	4.07	0.78		1.26					
Liver extract + kidney extract	20	2.10	3.88	1.01		1.31					24
	20	2.46	3.55	1.03	1.12	1.25	1.77				24
	20	2.02	3.64	1.38	1.70	1.47	1.92				24
											24
	20	2.18	3.78	1.54	1.68	1.85	1.74				
	20	2.50	3.52	0.72	1.53	1.67	1.81				24
	20	2.16	3.97	1.04		1.29	1.86				24
	20	2.13	3.58	1.00	1.45	1.00	1.65				4
	20	1.93	3.61	1.31	1.45	1.31	1.97				4
	14	0.86	1.97	1.40	1.52	1.40	1.96				3
	14	1.19	1.45	1.16	1.31	0.76	1.05				3
Liver extract, incubated with histidine + kidney homogenate	14	1.29	2.10	1.16		1.31	1.52				3
	14	1.40	2.14	1.05	1.42	1.21					3
	14	1.18	2.13	1.26		1.00	1.26				3
	14	1.00	2.30	1.05		1.05	1.47				3
	3	0.30		0.25				5.12	5.65	0.53	4
	3	0.32		0.13	0.19			2.99	3.24	0.25	4
	3	0.33		0.18	0.18			7.50	8.13	0.63	4
	3		0.51			0.24	0.35	5.50	6.04	0.54	4
	3		0.55			0.29	0.42	7.88	8.69	0.81	4
				In an atmosphere of N_2							
	14	1.05	2.10	0	0	0.79	1.40				3
	14	1.05	1.89	0	0	1.15	1.41				3
	14	1.17	2.57	0	0	1.31	1.47				3
Liver extract, incubated with histidine + kidney homogenate	14	1.45	2.86	0	0	0.70	1.42				3
	14	1.12	2.02	0	0	1.04	1.30				3
	3	0.26		0	0						4
	3	0.21		0	0						4
	3	0.21		0	0						4

The following schedule was followed in these experiments:

1) 20 mg of histidine dissolved in 4.5 ml of 0.1 M phosphate buffer, pH 8, and 4 g of kidney homogenate, were added to 7.5 ml of liver extract.

2) 20 mg of histidine and 8.5 ml of kidney extract, were added to 7.5 ml of liver extract.

3) 4 g of kidney homogenate was added to 12 ml of liver extract, previously incubated in a thermostat with 20 mg of histidine until its complete degradation, which was determined by the diazo reaction and the bromine test. Before the addition of kidney homogenate the liver extract was boiled for the removal of proteins.

In those experiments which were performed in Warburg apparatus we used the centrifugate from the incubated liver extract. The small flasks in which the experiments were carried out were tightly closed with rubber stoppers and kept in the thermostat at 38° for 24 hours. Chloroform was added as an antiseptic. The control flasks did not contain histidine. In experiments performed under anaerobic conditions the atmosphere was filled with nitrogen purified from traces of oxygen by passage through an alkaline solution of pyrogallol. 4 ml of 20% trichloroacetic acid was introduced into each flask for the precipitation of proteins. The ammonia and amino nitrogen were determined in the filtrate by the same methods that were used in experiments with liver extracts alone. The amino nitrogen in the filtrates was determined as usual after the removal of ammonia. The liver and kidney extracts were obtained by grinding these organs in a mortar with fine glass, then extracting for 10-15 min. with 3 times their volume of 0.1 M phosphate buffer, pH 8. The extracted tissue was separated by centrifugation.

For a more detailed investigation of the conditions of the enzymatic breakdown of the product of urocanic acid conversion in kidneys we also performed a number of experiments in the Warburg apparatus. 1.1 ml of Ringer-Krebs solution, pH 7.4-8, and 400 mg of kidney homogenate were introduced into the main vessel of the Warburg flask. 1 ml of incubated liver extract containing 3 mg of degraded histidine was introduced into the side-bulb. 1 ml of incubated liver extract without histidine was introduced into the side-bulb of the control flask. Respiration was observed for 4 hours. The proteins were precipitated by 1 ml of 14% trichloroacetic acid. As seen from the results of the experiments given in Table 3, the product of urocanic acid conversion, which forms in the liver from histidine as a result of the successive action of histidine deaminase and urocanase, is enzymatically split in kidney extracts and homogenates. It is known that the characteristic properties of the product of urocanic acid conversion are its stability to the action of a saturated solution of sodium carbonate and also to hydrolysis by a strong alkali (30% NaOH), which is accompanied by the release of a molecule of ammonia and the formation of an amino compound of a still unknown structure. However, after the action of the kidney enzyme under aerobic conditions on the product of urocanic acid conversion and even upon treatment of the trichloroacetic filtrates with a saturated sodium carbonate solution there is more than 1 molecule of ammonia liberated, and an amino compound is formed which in its properties resembles the substance detected upon hydrolysis of the product of urocanic acid conversion with strong alkali, since its amino nitrogen is fully determined in the Van-Slyke apparatus only with agitation for 10 minutes. Therefore, the alkaline and enzymatic degradation of the product of urocanic acid conversion occur in the same way and lead apparently to the formation of the same degradation products. This enzyme, characterized by a relatively large activity, effects degradation of 45-90% of the product of urocanic acid conversion estimated by the amount of amino nitrogen formed upon treatment of the reaction mixture by a saturated solution of sodium carbonate. However, it is necessary to point to the fact that upon enzymatic degradation of the product of urocanic acid conversion in kidney extracts or homogenates the formation of an amino group under aerobic conditions is not always accompanied by an equivalent displacement of ammonia by the saturated solution of sodium carbonate. The displacement of more than 1 molecule of ammonia by sodium carbonate upon the degradation of the product of urocanic acid conversion under the influence of kidney enzymes is observed only in experiments with a 24-hr. incubation. Upon a shorter incubation (3-4 hr.) in the presence of oxygen in the Warburg apparatus, despite the formation of an amino group, only one molecule of ammonia is displaced by sodium carbonate. It is difficult at the present time to find an explanation for this phenomenon.

It is very interesting that the degradation of the product of urocanic acid conversion in kidneys is accompanied by an absorption of oxygen and that it is completely stopped under rigorous anaerobic conditions (in an atmosphere of nitrogen). The enzyme system catalyzing the breakdown of the product of urocanic acid conversion is easily extracted from kidney homogenate by phosphate buffer optimally between pH 7.4 and pH 8.

DISCUSSION OF RESULTS

After the investigations performed in our laboratory and the confirmation of other authors, some of whom also employed isotope analysis, there is little doubt concerning the transformation of histidine into urocanic acid in the first stages of its biological conversion; however, there is no such assurance as yet of the mechanism of the degradation of urocanic acid and of the nature of its end-products. In previous works [2-3], we reported some convincing evidence on the complete inconsistency of the formation of glutamic acid from histidine or urocanic acid. The formation of glutamic acid from histidine was also not confirmed in experiments where ordinary histidine [13] or histidine labeled with the nitrogen isotope N^{15}, in position 3 of the imidazole ring [25], was administered to rats or in experiments where injections of histidine labeled with the carbon isotope, C^{14}, in the carboxyl group were given to the same animals. At the same time a number of works have been

reported describing the transformation of histidine into glutamic acid through the stage of urocanic acid formation upon degradation by bacterial enzymes of ordinary histidine [16] and of its various labeled preparations containing the isotope of nitrogen, N^{15}, in the amino group or in position 3 of the imidazole ring or the isotope of carbon, C^{14}, in position 2 of the same ring [17]. Analogous results were obtained upon intravenous injections in rabbits and upon the degradation of histidine, labeled with the C^{14} in the carboxyl group, in guinea pig liver slices [14]. According to the latest data, formamidinoglutaric acid [18] or formylglutamic acid [19] are found as intermediate products in the conversion of histidine into glutamic acid through the stage of urocanic acid formation.

In spite of this, it is apparent from our previous investigations and the results of our present work that the transformation of histidine into glutamic or any other amino acid does not take place in the liver. The amino compound is formed only upon enzymatic degradation of the product of urocanic acid conversion in kidneys or upon its hydrolysis with strong alkali. While the nature of this substance remains unknown, it most probably has one carboxyl group and its amino group is in the γ- or possibly in the δ-position. The possibility of formation of γ- aminocrotonic or γ-aminobutyric acids [20], upon enzymatic degradation of the product of urocanic acid conversion is not excluded.

The subsequent investigation of the biological conversion of the histidine molecule acquires a special interest when the utilization of the carbon in position 2 of the imidazole ring for the synthesis of purines, serines, the methyl and ethanolamine groups of choline, and the methyl group of creatine in normal [21-23] and tumor-affected animals [24] is considered. As previously, we failed to observe any relationship between the decrease of histidine degradation in liver and the size of the animal tumors. Histidine degradation is inhibited in the presence of a small developing blastoma to almost the same degree as in the presence of a tumor equal in weight to that of the whole body of the animal. This is probably due to a decrease in the synthesis of enzyme systems occurring in the earliest stages of development of the given tumors.

SUMMARY

The degradation of histidine in liver stops at the stage of formation of the product of urocanic acid conversion. The latter is apparently transformed under aerobic conditions into γ-aminocrotonic or γ-aminobutyric acid under the influence of a special enzyme system found in kidneys, similarly to the action of alkali. The degradation of histidine in the liver of rats with muscle tumors is less intensive than in liver of healthy animals.
In conclusion, I would like to express my gratitude to Professor A. N. Parshin for his valuable suggestions.

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CYCLOPEPTIDES AND COMPOUNDS OF THE AMIDINE TYPE IN THE INTERMEDIATE PRODUCTS OF ACID CASEIN HYDROLYSIS

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The structure of proteins, while being one of the fundamental problems of biological chemistry, represents at the present time one of the unsolved questions of the chemistry of high molecular natural compounds.

According to the theory of Zelinski and Gavrilov [1] the basic structural unit of the protein molecule is amidine. In determining the structure of the protein molecule Gavrilov and his co-workers [1-5] applied the quantitative characteristics of the absorption spectra of biuret complexes of proteins, amidines and peptides, as well as other methods. They demonstrated [4] that the absorption spectra of biuret complexes of peptides and those of amidines and proteins differ in their absorption maxima and intensity of absorption.

It is also known that the intensity of the biuret reaction for proteins and peptides is approximately the same when equal amounts of material are considered [6]. For the products of protein hydrolysis it must be proportional to the amount of peptide nitrogen if the hydrolysis product is a mixture of polypeptides. In the presence of large amounts of diketopiperazines which do not give a biuret reaction, the reaction must be weak in spite of the high content of peptide nitrogen. Taking into consideration the fact that the absorption spectra of amidine (piperazine-bis-diglycylglycyl ester) is of the tripeptide type [5] it must be concluded that, even in the presence of compounds of the amidine type in the products of protein hydrolysis, the same inconsistency should be observed upon total hydrolysis of the material between the amount of peptide nitrogen calculated, on one hand by the intensity of the biuret reaction and, on the other, by the increase of amino nitrogen. Sild [7], following the recommendations of L. T. Solovyev, compared the intensity of the biuret reaction in the electrophoretic fractions obtained from the products of the terminal degradation of fibrin by pepsin with the peptide nitrogen content. She demonstrated that no detectable amounts of diketopiperazines composed of monoamino acids were present in these products.

In the present work we also compared the intensity of the biuret reaction in the electrophoretic fractions obtained from the products of partial acid hydrolysis of casein with the amount of peptide nitrogen. This allowed some conclusions to be formed on the nature and size of the particles constituting the intermediate products of casein hydrolysis.

Analytical Methods

We used commercial casein precipitated 3 times by 0.1 N HCl from a 0.1 N NaOH solution. The total nitrogen of such casein was 15.45%, and the ash content: 1.86%.

The hydrolysis was performed in the vapors of a water bath with reflux condenser in 1 N H_2SO_4. The initial casein was heated for 5.5 hours (1st stage of hydrolysis); the hydrolysate was filtered, the residue carefully washed with distilled water and further hydrolyzed under the same conditions for 11 hours (2nd stage). The insoluble residue from the second hydrolysate was hydrolyzed for 22 hrs. (3rd stage) and the insignificant residue from the latter for 44 hrs. (4th stage). The following amounts of 1 N H_2SO_4 were taken per 1 g of nitrogen: 78 ml for the 1st, 115 ml for the 2nd, 147 ml for the 3rd and 214 ml for the 4th stage of hydrolysis.

The initial casein, the intermediate products of Stages I-IV, the fractions of the neutral, acid and alkaline products obtained by electrophoretic separation of the hydrolysates from Stages I-IV were also totally hydrolyzed

(100°, 24-37 hrs, 30 ml of 5 N H_2SO_4 per 1 g of nitrogen). Due to insignificant amount of material left, only the neutral fraction (C) from the products of Stage IV was taken for the total hydrolysis. The sulfuric acid was removed before electrophoresis, by the addition of barium oxide, up to pH 3-4, to the hydrolysates. The residue was washed several times with hot water, the filtrate steamed under vacuum and added to the basic solution. After precipitation of the main bulk of sulfuric acid as $BaSO_4$ in the Stage I hydrolysate up to pH 3, and after the washing of the residue for more than 30 times with distilled water, 12.4% of the nitrogen used in the experiment was lost. Losses in the hydrolysates of Stages II, III and IV were halved by washing the residue 3-4 times with a hot solution of 0.1 N HCl, distilling the latter under vacuum, and adding the extract to the main filtrate.

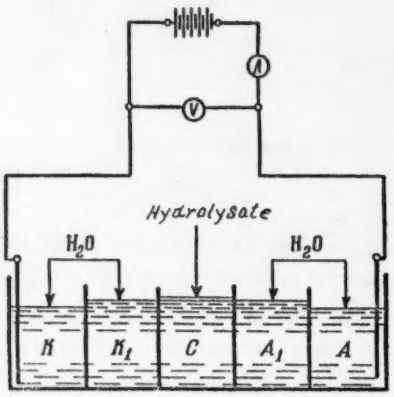

Fig. 1. Diagram of the electrophoresis of intermediate hydrolysates of Stages I-IV.

The electrophoresis of the neutralized hydrolysates of Stages I-IV was carried out in an apparatus consisting of 5 cells. We used chromogelatin membranes on linen. The cathode was a fine copper wire mesh; the anode – a fine platinum wire mesh. The intensity of the direct current was 110-120 V. The hydrolysate was introduced in the middle cell while the other cells contained distilled water. The solutions in the cells will be represented for convenience by the following designations: 1) Fraction A or the acid fraction; 2) Fraction A_1 or the weak acid fractions; 3) Fraction C or the neutral fraction; 4) Fraction K_1 or the weak alkaline fraction; 5) Fraction K or the alkaline fraction (Fig. 1).

At the beginning of electrophoresis the electrodes were placed in the K_1 and A_1 compartments. As soon as the intensity of the current began to increase they were transferred to the distal compartments. The fractionation was stopped when the current intensity dropped to 0.04 amp and the electrical conductivity of the K_1, C and A_1 solutions approached the electrical conductivity of 0.0002-0.001 N KCl solutions. The electrophoresis of the Stage I hydrolysate lasted for 145 hrs., Stage II – 60 hrs., Stage III – 37 hrs., and Stage IV – 13 hrs.

The total nitrogen was determined by the micro-modified method of Kjeldahl. The determination of amino nitrogen was carried out by titration with formol according to Northrop. The ammonia nitrogen was determined according to Folin.

For the determination of the intensity of the biuret reaction a 12% solution of KOH was added to the solution up to a final 3% concentration of KOH, then 1 ml of a 20% $CuSO_4$ solution, and the volume of the mixture brought up to 25 ml with 3% KOH. The mixture was strongly agitated for a long time and after 10 min. was centrifuged for 5 min. The extinction of the clear solution was determined on the Pulfrich photometer with a cuvette 30 mm thick and the filters S = 530 and 570 mμ. In those cases where the absorption maxima did not lie within these two wavelengths the photometric measurements were made with other (adjacent) filters.

The peptide nitrogen was calculated according to the following formula:

$$\frac{\text{amino nitrogen of the total hydrolysate} - \text{amino nitrogen of the intermediate hydrolysate}}{\text{total nitrogen}} \times 100,$$

where the total and amino nitrogen are corrected for ammonia.

Ammonia, Amino and Total Nitrogen Contents of Intermediate Products of Hydrolysis

As seen from Table 1, about 2/3 of the total solid matter taken for hydrolysis goes into solution at each step of the degradation during the hydrolysis of casein in 1 N H_2SO_4. The nitrogen content of the soluble products of the 1st stage is 58.7%, that of the 2nd stage – 26.5%, and that of the 4th stage only 4.1% of the total nitrogen

contained in the soluble products of all 4 stages. The remainder (0.27 g of nitrogen) from the 4th hydrolysate was rejected.

The ratio of the amino nitrogen to the total nitrogen was the smallest in the products of the Stage I hydrolysate and the greatest in Stage IV. In the former the ratio is almost the same as in the case of the total digestion of casein by pepsin (Sild [7]). The ratio of the amino nitrogen to the total nitrogen is usually taken as a measure of the average size of the particles. It follows from Table 1 that the size of the particles decreases as the hydrolysis proceeds. However, even after an 82.5 hr. hydrolysis of the insoluble residue (Stage IV) the solution contained substances, usually of a dipeptide nature, in which the content of amino nitrogen was 43.2%.

TABLE 1

General Characteristics of the Products of Casein Hydrolysis

Stage of hydrolysis	Duration of hydrolysis (hours)	Total nitrogen (g)	Amino nitrogen (mg)	Ammonia nitrogen (mg)	Amino nitrogen, in % of total nitrogen (without ammonia)
I	5,5	13.41	3680	1840	15.9
II	11	5.38	1433	163	24.3
III	22	2.14	651	12	30.0
IV	44	0.80	349	7	43.2
Residue		0.27			

91% of the ammonia is split off after a 5.5 hr. hydrolysis of casein in 1 N acid. Upon further hydrolysis of the insoluble residue the increase in ammonia is insignificant. Upon the action of a stronger (5N) acid on the intermediate products of hydrolysis, an increase in the amount of ammonia is observed and at each stage the increase in ammonia consists approximately of 1.6% of the total nitrogen of the intermediate products of hydrolysis. The amount of ammonia split off upon total hydrolysis of the intermediate products of Stages I-IV consists of 10.97% of the total nitrogen, and in the total casein hydrolysate – 10.4%.

The nature of the products or bonds breaking down upon the action of strong acid remains obscure. The increase in ammonia upon total hydrolysis may be due to the degradation of tryptophan and partially to that of hydroxy amino acid [8].

It should be pointed out that the protein which dissolved during the 5.5 hr. heating in 1 N H_2SO_4 differs from the initial casein in that the greatest part (91%) of its acid-sensitive amide groups are destroyed.

Distribution of the Intermediate Products of Casein Hydrolysis Among the Electrophoretic Fractions

On the basis of the data in Table 2 one may conclude that the substances of weak acidity, belonging to the neutral fraction, predominate in the bulk of intermediate products of casein hydrolysis as well as in the products obtained at the various stages of its hydrolysis. This is especially apparent at the 1st stage of hydrolysis. The amount of these substances significantly decreases at the 2nd stage and later on does not change any more. There are 45% less acid products (Fractions A_1 + A) and alkaline products (Fraction K + K_1), judging by the amount of nitrogen, in the Stage I hydrolysate than in the hydrolysates of Stages II, III and IV. Judging by the amount of nitrogen present, there are approximately 10% of acid products and 22.5% of alkaline products present in the latter stages.

There are two possible explanations of such a distribution of nitrogen products among the fractions at the 1st stage of hydrolysis. The most probable explanation is that during the 5.5 hr. hydrolysis of casein, fewer linkages are formed which result in acid substances that are negatively charged at pH 4.5-5.5, than alkaline substances which are positively charged at the same pH. The other possible explanation, that the migration of large charged particles into the distal cells is hampered by the size of the pores of the chromogelatin linen membranes, is inconsistent, since during the investigation of the products of the hydrolysis of other proteins some cases were observed where negatively charged particles migrating to the pre-anode compartment were of the same average size as the particles of Fraction C of the Stage I hydrolysate [7].

TABLE 2

Nitrogen Content in the Various Fractions of the Intermediate Products of Casein Hydrolysis

Stage of hydrolysis	Nitrogen, in % of the sum of the nitrogen in the fractions (without ammonia)					Sum of the nitrogen in the fractions (mg) (without ammonia)
	K	K_1	C	A_1	A	
I	7.19	3.14	86.31	1.46	1.9	2539
II	16.10	6.25	67.74	3.95	5.96	2450
III	13.40	9.10	67.80	3.2	6.50	963
IV	15.65	8.80	67.20	5.19	5.16	341
In % of the sum of the nitrogen in Stages I-IV	10.62	4.73	78.57	2.47	3.61	100
pH of the solutions	~10	6.5	4.5—4.2	4.2—4.0	3.2	

Thus, our qualitative information regarding the electrochemical properties of the intermediate products was uniform at all stages of the degradation of proteins, while the quantitative relationships among the fractions were not always the same. The stage of least hydrolysis (I) is especially noticeable in this respect.

The ratio of the amino nitrogen to the total nitrogen increases with stronger hydrolysis, as seen from Table 3; this ratio is especially high in the products of the pre-anode fractions, relatively high in neutral fractions, rather small in alkaline fractions, and practically constant in acid fractions. At the early stage of hydrolysis (I) the average size of the particles of the acid fraction is significantly smaller than that of the neutral fractions. Upon further hydrolysis this difference decreases; for example, at the 3rd stage of hydrolysis, the highest ratio of the amino nitrogen to the total nitrogen (Fraction A) is greater than the smallest ratio (Fraction C) by an approximate factor of 2.5, while at the 1st stage of hydrolysis this ratio of the same fractions differs by a factor of 4.5.

Losses of Material Upon Neutralization of the Hydrolysates and During Their Electrophoresis

Upon neutralization of the sulfuric acid by barium oxide in hydrolysates of Stages I and II a significant loss of ammonia occurred (16.3% at the 1st and 41.1% at the 2nd stage, of the initial amount of ammonia present). The losses of nitrogen products not belonging to ammonia were relatively small (3.0, 2.2 and 4.6% of nitrogen in hydrolysates of Stages II, III and IV respectively). Significant losses of nitrogen products (12.4%) were observed only upon neutralization of the Stage I hydrolysate, and the amount of compounds containing phosphorus decreased almost to the same extent (13%). In other words, either a part of the hydrolysate obtained after a 5.5 hr. hydrolysis of casein in 1 N H_2SO_4 was adsorbed by the barium sulfate residue, or the latter was insufficiently washed.

Similar difficulties were encountered upon fractionation of the hydrolysates by electrophoresis. It is true that the losses were relatively small. However, they complicated our studies of the intermediate products under investigation. We consider as "losses", the discrepancy between the sum of nitrogen determinations in various fractions after electrophoresis and the determination of nitrogen in the total hydrolysate. Actual losses and unavoidable errors of determination must be accounted for in this discrepancy. In the presence of voluminous fractions the latter may represent a significant part of the discrepancy.

The smaller the average size of the particles separated by electrophoresis in a multicellular apparatus, the smaller is the loss of nitrogen. The maximum loss occurred in the Stage I hydrolysate (7.2%) while no losses were observed in the Stage IV hydrolysate. Apparently, this phenomena is primarily due to changes in the pH of the solution and to the shift of the latter toward the isoelectric point at which the solubility of the ampholyte is

is minimal. The compounds separating in the form of a fine suspension are partly deposited as a dense layer on the membranes and partly coagulated. For this reason, the losses may be even greater upon an insufficient cleaning of the apparatus.

TABLE 3

Average Size of Particles in Intermediate Products of Casein Hydrolysis in 1 N H_2SO_4

Intermediate hydrolysis	Amino nitrogen, in % of total nitrogen (without ammonia)		Peptide nitrogen, in % of total nitrogen		Mean value of R in particles		
	At the intermediate stage	Upon total hydrolysis	From the amino nitrogen determinations	According to the intensity of the biuret reaction	According to the increase in amino nitrogen	According to the intensity of the biuret reaction	According to the increase in amino nitrogen corrected for the terminal NH_2-group of lysine
Stage	15.7	80.8	65.1	65.9	5.15	5.20	7.0
	22.4	82.1	59.7	57.8	3.66	3.60	4.3
	28.4	82.1	54.1	57.8	2.90	3.03	3.2
	42.2	83.0	40.8	40.0	1.97	1.92	2.1
Fraction							
Stage	24.2	70.6	46.4	45.5	2.92	2.88	4.8
	23.8	63.0	39.2	39.4	2.64	2.66	4.4
	32.4	64.0	32.4	32.6	1.97	2.00	2.5
	32.8	—	—	38.6	—	2.18	2.8
Fraction							
Stage	44.6			39.5		1.90	2.1
	24.8			40.1		2.62	3.2
	27.7			45.0		2.62	3.2
	33.6			(45.8)		2.46	2.6
Fraction							
Stage	14.0	83.6	69.6	57.6	5.96	5.83	7.3
	19.2	80.9	61.7	64.2	4.20	4.34	4.8
	25.1	78.0	52.9	53.5	3.10	3.13	3.3
	41.5	84.4	42.9	45.6	2.03	2.06	2.1
Fraction							
Stage	22.7			(85)		4.74	
	36.2			56.5		2.56	
	62.4			39.0		1.60	
	75.0			(14.0)		1.20	
Fraction							
Stage	63.6	90.0	26.4	(143)	1.4		
	54.6	83.7	29.2	(44.8)	1.53		
	66.6	83.7	16.7	(29.7)	1.25		
	66.0	—	—	—	—		

The lack of nitrogen is to a certain extent due to the deamination of amino acids and peptides on the anode. This was observed by us upon fractionation of total as well as partial hydrolysates (in Fraction A the amount of ammonia nitrogen, as % of the total nitrogen of the fraction, was: at Stage I – 18.5; Stage II – 3.8; Stage III – 4.4 and Stage IV – 2).

Absorption Spectra of the Biuret Complexes of Casein and of the Main Fractions of Its Hydrolysis Products

The above data does not give precise information on the size and nature of the particles forming upon partial hydrolysis of casein. In order to solve the problem of the size of these particles it is necessary to know at first approximation, the position of the maximum of the absorption spectra of the biuret complexes of these particles.

As shown by the photometric data, the absorption maxima of the biuret complexes of the non-fractionated intermediate products of StagesI-IV differ from each other. For the hydrolysates of Stages I-III, the absorption maxima are found within the wavelengths, between 530-570 mμ, and for the Stage IV hydrolysate it is shifted toward the longer wavelengths.

However, the intermediate hydrolysates of each stage represent to some extent a polydispersoid mixture. This can be easily seen if the absorption spectra of the biuret complexes of the intermediate products of the electrophoretic fractions of each stage are measured. Figure 2 presents the absorption curves of the biuret complexes of the intermediate products of the Stage III hydrolysate, of its main fractions (K, C and A), and of casein.

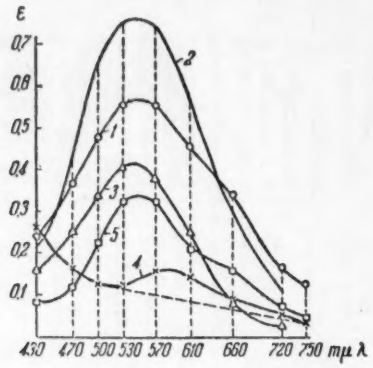

Fig. 2. Absorption curves of the biuret complexes of the intermediate products of the Stage III hydrolysate, its fractions and casein:
1) Stage III hydrolysate; 2) fraction C; 3) fraction K; 4) fraction A; 5) casein.

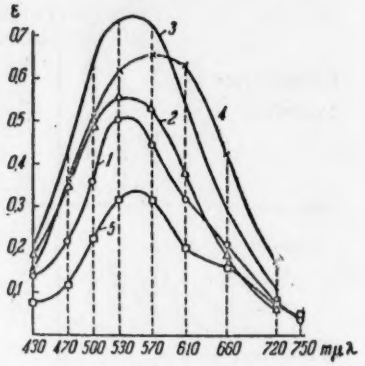

Fig. 3. Absorption curves of the biuret complexes of casein and of the intermediate products of the C fractions of the hydrolysates of Stages I-IV:
1) Stage I; 2) Stage II; 3) Stage III; 4) Stage IV; 5) casein.

The fact that the absorption maximum of the biuret complexes of the non-fractionated Stage III hydrolysate coincides with the absorption maximum of the products of its Fraction C and with the absorption maximum of casein is noteworthy. They are all found at the 550-560 mμ wavelength which is characteristic of the absorption spectra of particles of an average tripeptide size; the violet color of the biuret solution is the same for all of them. The absorption maximum of the biuret complexes of the alkaline products of the Stage III hydrolysate is insignificantly shifted toward the red side and is found at the 540-545 mμ wavelength, i.e. the wavelength close to the absorption spectra of tetrapeptides. The color of the biuret solution is cherry-violet. The same color is produced by the solutions of the cathode fractions of the hydrolysate of all the remaining stages. It must be noted that the color of the biuret solution is to a certain extent dependent also on the amino acid composition of the peptides and not only on their size (for instance Fraction K of Stage III).

Only the absorption maximum of the acid products (Fraction A) is significantly shifted toward the region of longer wavelengths (590 mμ), characteristic of dipeptide. We will also note here that the products of the A fractions of the remaining stages and that the A_1 fractions of the hydrolysates of Stages III and IV produce a blue biuret reaction and have their absorption maximum in the interval of 570-610 mμ.

The absorption curves of the biuret complexes of the intermediate products of the remaining non-fractionated hydrolysates (Stages I, II and IV) have, as in the case of the Stage III hydrolysate, an absorption maximum which coincides with that of the biuret complexes of the corresponding C fractions. The spectrophotometric curves of the latter are presented on Fig. 3.

The absorption maximum of the products of Fraction C of the Stage I hydrolysate and therefore, of the non-fractionated hydrolysate itself, is found at the 535-540 mμ wavelength. This wavelength is especially characteristic of heptapeptides [4] as well as of a mixture of tetra- and tripeptides.

The absorption maximum of the biuret complexes of Fraction C of the Stage II hydrolysate is shifted even more toward the red side in comparison to the above described Fraction C and is found at the 530-535 mμ wavelength. The absorption maximum of Fraction C of the Stage IV hydrolysate is, on the contrary, significantly shifted toward the longer wavelengths (590 mμ).

The absorption maxima of the biuret complexes of the intermediate products of the alkaline fractions (K) are found in the region of short wavelengths (520-540 mμ) for the hydrolysates of Stages I and II and at the 560-590 mμ wavelength for the Stage IV hydrolysate. The absorption maxima of all the intermediate products of the K_1 fractions are found in the same band (560-590 mμ). The absorption curves of their biuret complexes are not given.

Therefore, the conclusion that low molecular peptides, usually dipeptides, are found only in the Stage IV hydrolysate and in the A fractions of Stages I-III and in the A_1 fraction of Stage III, should be made on the basis of the shape of the absorption curves of the biuret complexes.

While no substantial difference in the absorption spectra of the biuret complexes of the products of the alkaline, neutral, and partly acid A_1 fractions (Stage I and II hydrolysates) could be detected, no definite conclusion on the size and especially on the nature of the particles contained in them could, however, be reached solely on the basis of their absorption maxima. Therefore, we deemed it necessary to correlate the results of the determinations of peptide nitrogen, made on the basis of the intensity of the biuret reaction, with those determinations that were made on the basis of the increase in amino nitrogen upon total hydrolysis of the intermediate products of the fractions.

For the determination of the peptide nitrogen on the basis of the biuret reaction, we plotted a standard curve. The solution of the C fraction of the Stage I hydrolysate was taken as the standard solution since it is the closest to peptones in its composition and particle size. In order to determine the standard curve various amounts of peptide nitrogen from Fraction C were taken and, after their treatment as described above, the obtained biuret complexes were observed photometrically at the 530 mμ wavelength (maximum extinction). The extinction was plotted on the ordinate and the peptide nitrogen on the abcissa. A linear relationship between the extinction and the peptide nitrogen was found (Fig. 4).

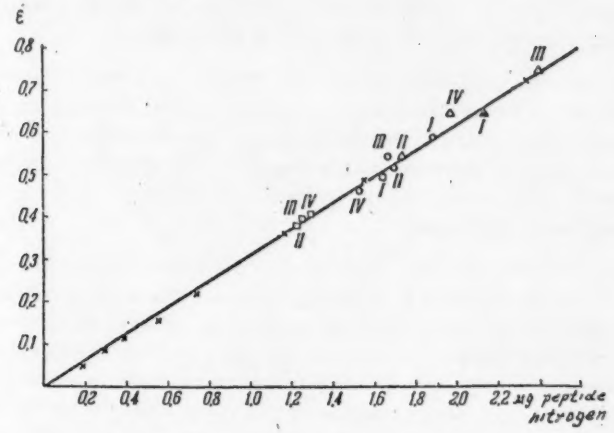

Fig. 4. Standard curve for the determination of peptide nitrogen: Extinctions of: x) standard solution; Δ) Fraction C; □) Fraction K; O) non-fractionated hydrolysates; experimental conditions: volume of solution – 25 ml; width of the cuvette – 30 mm; filter S = 530 mμ.

Upon correlating the obtained data on the amount of peptide nitrogen determined on one hand by the increase in amino nitrogen and on the other by the intensity of the biuret reaction in the neutralized hydrolysates of Stages I-IV, in the products of the C fractions of Stages I-IV and the K fractions of Stages I-III, a good agreement was found.

For better comparison, the amounts of peptide nitrogen and the corresponding extinctions were plotted on the same diagram with the calibrating curve (Fig. 4). As seen from Fig. 4 the points of intersection of all intermediate products, except those of the Stage III hydrolysate and of the C fraction of Stage IV, are found to be almost on the standard curve. Therefore, the latter may be applied for the determination of the peptide nitrogen in the products of Fractions K_1 and A_1. The reason for the deviation of the amounts of peptide nitrogen in the

intermediate Stage III hydrolysate and Fraction C of Stage IV is difficult to determine at the present time. The given standard curve may not be applied for the most acid products. Since the solutions of the A fractions are stained yellow in the presence of aromatic substances the extinctions become, as a rule, greatly raised. This is clearly seen from the absorption curves of the biuret products of Fraction A of the Stage III hydrolysate (see Fig. 2).

DISCUSSION OF RESULTS

On the basis of the agreement of the amounts of peptide nitrogen computed by two different methods (Table 3) we come to the conclusion that no detectable amounts of free diketopiperazines or compounds of the amidine type are present in the intermediate products of the hydrolysis of casein in 1 N H_2SO_4. They could not have been in the material lost upon neutralization since washing of the barium sulfate residue with methyl and ethyl alcohols in various concentrations did not lead to the detection of any nitrogen-containing products. The washing with 0.05 N HCl of the $BaSO_4$ residue from the Stage I hydrolysate resulted in the recovery of half of the lost nitrogen (repeated 4 times). The ratio of the amino nitrogen to the total nitrogen was the same in these products as in the initial hydrolysate. Free diketopiperazines were not detected upon acid hydrolysis and in other experiments performed in our laboratory [7]. According to Poroshin [5] no more than 1.36-1.42% of the total nitrogen is found in free diketopiperazines upon digestion of casein by pepsin in 0.1% HCl. However, the author considers that the presence of amidines in the products of hydrolysis is possible.

It is possible to compute the average size of the particles contained in the intermediate products, by using the standard curve and the amount of amino nitrogen. These data are given in Table 3. By comparing the mean value R (the number of amino acid residues) in particles, determined by the two methods, it is apparent that they agree quite well.

It must be noted that the mean value R in particles was not corrected for the ϵ-amino group of lysine. Upon correction for the probable content of lysine in hydrolysates, the ϵ-amino group of which is determined by formol titration, the mean value R in the products of Fractions K_1, K and C and also of the non-fractionated hydrolysates of Stages I-IV, will be greater. In this case the size of the particles is in good agreement with the absorption maximum of the biuret complexes of the corresponding fractions.

As a rule the size of the particles decreases as hydrolysis proceeds. It is not so apparent in the hydrolysates of Fractions K, K_1 and A, while in the products of Fractions C, A_1 and in the non-fractionated hydrolysates, this decrease is quite distinct: upon further hydrolysis the size of the particles decreases by a factor of 1.5-2. If the dispersion of the particles is compared at various stages then, as a rule, the size of the particles of the various fractions differ from each other by a factor of 2 or 3, and seldom by a factor of 4 or 5. In other words, the protein molecule is split into particles of similar size.

If the peptide nitrogen of casein is computed on the basis of the intensity of the biuret reaction and on the basis of the amino nitrogen, it is found that there is a close agreement between these two values. Thus, the amount of peptide nitrogen determined by the intensity of the biuret reaction (ϵ, maximum at the 530 and 570 mμ wavelengths, is equal to 0.313) was found to be 1.00 mg. An amount of casein containing 1.4 mg of nitrogen was taken for photometric measurements. If 5% of this nitrogen, found in the terminal amino group, is subtracted and if the fact that the amino nitrogen represents 80% of the total nitrogen of the total hydrolysate of casein is taken into consideration, the peptide nitrogen must then equal 1.06 mg. This value is obtained under the condition that the amide nitrogen plays a role in the formation of the biuret complex, of which there is indirect evidence [2].

Thus, on the basis of our data, we come to the conclusion that compounds of a diketopiperazine nature are probably absent from casein and if there are any, their amount must be small.

In conclusion we would like to note that the use of the biuret reaction has an important significance in the analysis of the complex mixture of the intermediate products of protein hydrolysis but that it still has to be considerably perfected on pure peptides, amidines and on their mixtures with various amino acid compositions.

SUMMARY

91% of the ammonia formed upon heating of casein in 1 N H_2SO_4 is split off during the first 5.5 hrs. and the degradation of casein is practically completed after the following 11 hrs. of hydrolysis. The ammonia nitrogen represents 10.4% of the total mass of nitrogen in the intermediate products.

Upon separation of the intermediate products of casein hydrolysis by the method of electrophoresis in multicellular apparatus with membranes, the weak acid (neutral) fraction represents the main bulk of the products, while only a small amount enters into the acid fractions. The amount present in the neutral fraction of the first stage of hydrolysis is especially large (86.3%).

The intermediate products of the acid hydrolysis of casein are substances of a peptide nature.

The average particle size is the greatest in the neutral fraction and the smallest in the acid fraction; at its 1st stage, the latter already contains free dicarbonic amino acids together with dipeptides. Only a small portion of their total protein content is split off in the form of dicarbonic amino acids and low molecular peptides. As hydrolysis proceeds the average size of the particles in the alkaline fraction decreases; that of the neutral fraction decreases much and that of the acid – relatively little.

There is no ground to assume a large polydispersion of the particles at all stages of hydrolysis.

I would like to express my gratitude to Professor G. E. Vladimirov for his suggestions given during the course of this work

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SYNTHESIS OF NICOTINE-C^{14} IN TOBACCO SEEDLINGS

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Investigations of tobacco alkaloids have shown that nicotine is formed upon the germination of tobacco seeds and that its synthesis occurs in the roots of the tobacco plant [1-4]. However, the pathways of nicotine biosynthesis are not yet ascertained and the function of the alkaloids in the life of the plant remains unknown; therefore, the perception of this phenomena as a whole presents a problem of a general biological interest.

According to published data, labeled nicotine was obtained by exposure of the tobacco plant during all of its vegatative stages and also of isolated tobacco leaves to an atmosphere of $C^{14}O_2$ [5, 6]. Of significant interest are the experiments on the introduction into the tobacco plant of labeled compounds of methionine, choline, glycine and formic acid as sources of the methyl group of nicotine. These investigations resulted in the positive determination that the methyl group of methionine and choline, and the carbon of glycine and formic acid are incorporated into the N—methyl group of nicotine [7-10]. An experiment with labeled nicotinic acid and tryptophan as precursors of nicotine, has shown that they do not act as such [4, 11]. Essentially these investigations did not uncover the mechanism of the biochemical process occurring in the plant and leading to the formation of the alkaloid nicotine. In order to investigate further this process in tobacco plants we decided to apply tracer techniques to obtain labeled nicotine in order to determine the conditions of its formation, and to follow the pathways of its conversion.

We used $NaHC^{14}O_3$ in a concentration of 48.7 mg per ml and with a specific activity of 2.2 mcurie/ml as the source of labeled carbon.

The experiment was carried out on tobacco seedlings in light and darkness at 18-20°. 5 g of tobacco seeds of the "Trapezond 93" variety were placed on filter paper in a Petri dish and moistened with Knoop nutrient mixture diluted 1: 2 and containing $NaHC^{14}O_3$ at pH8.0. Later on, the same solution was periodically added in amounts necessary to create the optimum humidity for the germination of the tobacco seeds. The radioactivity of the solution in each sample of the mixture was 20-25 μcurie in 25 ml of solution.

The nicotine was separated from the seedlings by steam and determined in the distillate by the method of Pfil — by precipitating the nicotine with picric acid. The obtained yellow crystalline powder of nicotine dipicrate was identified by its melting point. The radioactivity of the preparations was determined with an end counter of the T-25-BFL type with a mica window having a density of 1.3 mg/cm^2.

The obtained data representing the radioactivity of the isolated nicotine dipicrate are expressed in counts per minute (cpm) per 1mg of sample (Table 1).

The experiments on tobacco seedlings carried out in light and darkness on a nutrient mixture containing $NaHC^{14}O_3$ have shown that the formation of labeled nicotine occurs during the germination of tobacco seeds.

We used solutions with activities of 22 and 44 μcurie in order to determine the relationship between the biosynthesis of labeled nicotine and the concentration of labeled carbon dioxide. The experiment lasted for 10 days. The obtained results on the radioactivity of the seedlings and nicotine dipicrate are given in Tables 2 and 3.

TABLE 1

Radioactivity of Nicotine Dipicrate (Melting Point: 218°)

Conditions of exposure	Radioactivity of the added solution (μ curie)	Total cpm in dipicrate	cpm/1mg of nicotine dipicrate	cpm/1mg of nicotine	cpm/mM of nicotine $\times 10^4$
In light	5.0	92±3.2	18	70	1.13
	5.2	96±3.2	19	73	1.17
In darkness	4.6	97±3.2	21	81	1.31

TABLE 2

Radioactivity of Seedlings

Conditions of exposure	Amount of C^{14} (μ curies)	Sample of seedlings (mg of dry substance)	Total radioactivity (cpm)	Radioactivity in cpm/mg of dry substance
In light	22	6.0	479±5.8	80
	44	6.6	1046±6.5	158
In darkness	22	6.2	508±4.5	82
	44	8.0	783±8.1	98

A definite trend may be noted in the incorporation of CO_2 into the seedlings. The radioactivity of the seedlings is 5-7 cpm per mg on the 5th day and reaches 80-100 cpm per mg at the end of the experiment (on the 10th day). At the same time, an increase in the concentration of labeled carbon dioxide causes a corresponding increase in the radioactivity of the seedlings, in light; however, no such increase is observed in darkness.

TABLE 3

Radioactivity of Nicotine Dipicrate (Melting Point: 218°)

Conditions of exposure	Radioactivity of the added solution (μ)	Sample of nicotine dipicrate (mg)	Total radioactivity (cpm)	cpm/1mg of nicotine dipicrate	cpm/1mg of nicotine	cpm/mM of nicotine $\times 10^6$
In light	22	5.4	114±4.2	21	81	1.3
Ditto	22	2.0	46±3.8	23	88	1.4
In light	44	3.4	154±4.1	45	171	2.8
Ditto	44	4.4	189±4.5	43	163	2.6
In darkness	22	1.6	29±2.9	18	69	1.1
Ditto	44	5.6	116±4.5	20	76	1.2
Ditto	44	3.7	101±3.8	28	106	1.7

The radioactivity of the preparations of nicotine dipicrate, obtained from green and etiolated seedlings, shows that in light the intensity of the biosynthesis of nicotine labeled with C^{14} increases proportionally when the concentration of $C^{14}O_2$ is doubled, while it remains at the same level in darkness. Apparently, the excess of $C^{14}O_2$ entering the leaves is not used for the synthesis of the organic substance due to the lack of the necessary source of energy.

The green and etiolated tobacco seedlings display the ability to biosynthesize labeled nicotine using the labeled carbon dioxide from the solution as a source.

During our experiments we observed the absorption of radioactive carbon from sodium bicarbonate and its subsequent incorporation into the nicotine molecule. This phenomena is of exceptional interest in the light of the investigations on the use of carbon dioxide by the root system [12-15], which have shown that tobacco seedlings are able to absorb carbon dioxide from the solution. The latter, entering the cycle of biochemical conversions, is also incorporated into the product of the second biosynthesis – the alkaloid nicotine.

SUMMARY

The absorption of carbon dioxide from a nutrient solution containing $NaHC^{14}O_3$ by tobacco seedlings has been determined by the tracer technique.

The absorbed carbon dioxide is incorporated into the chain of biochemical reactions which take place upon the germination of tobacco seeds: nicotine-C^{14} was isolated, and its synthesis in tobacco seedlings under light and darkness was demonstrated.

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THE ROLE OF PHOSPHORYLATION IN THE ABSORPTION OF AMINO ACIDS IN THE INTESTINE

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According to published data [1] the absorption of amino acids in the intestine is assumed to be an active process and not only a simple diffusion. Some observations [2-6] which have shown that the l-amino acids are absorbed significantly better than the d-isomers also favor this assumption; glutamic and aspartic acids are absorbed significantly less than other amino acids [4] etc. During the investigation of the mechanism of amino acid absorption it was found [7] that free NH_2- and COOH-groups participate in the adsorption of amino acids and that the position of these groups does not influence the rate of absorption (α- and β-alanine are absorbed at the same rate). Some processes, the nature of which is still unknown, occur in the intestinal wall during the absorption of amino acids.

As shown by Kamin and Handler [8] some amino acids inhibit the absorption of other amino acids. These authors surmise that the absorption occurs by an enzymatic mechanism similar to the phosphorylation of carbohydrates in the intestine. It has been established by Verzar [9] that adrenalectomy disrupts the phosphorylation of carbohydrates which, as we know, is coupled with the absorption of the latter in the intestine. During his studies on the absorption of amino acids in normal and adrenalectomized rats, Laszt found [10] that the absorption of glycocoll is stimulated by the adrenal glands.

This made it possible to assume that phosphorylation plays a role in the process of amino acid absorption. Since this assumption needed experimental verification, we decided to carry out the present investigation.

EXPERIMENTAL

The investigation was begun with a series of experiments on rats with ligatures applied at the beginning and at the end of their small intestine.

The rats (numbering 20) were separated into three equal groups *. A mixture of amino acids: histidine, lysine, alanine, glycocoll, glutamic acid, cystine, and tyrosine (2 ml) was introduced with a syringe into the intestines of the animals of the first group. The total concentration of the amino acids was 0.5 M. The same solution, with the addition of 25 mg of inorganic phosphate, pH 7, was administered to the rats of the second group. The rats of the third group received into the intestine 2 ml of the same solution of amino acids with the addition of various amounts of phosphate (36, 80, 125 mg) as well as various amounts of ATP (80, 125, 250 mg).

30 or 60 minutes after the administration of the mixture of amino acids, the intestine was removed, its contents were washed out with distilled water and the contents of nitrogen, inorganic phosphate, labile and acid-soluble phosphorus were determined in the intestine, in the intestinal contents, and in the intestinal wall. The amount of absorbed amino acid nitrogen was computed by the difference between the nitrogen of the administered mixture and the amount of nitrogen recovered in the intestinal contents.

The results of the experiments with a 30 minute interval are given in Table 1 (average of 5-6 experiments of each kind), from which it is seen that the absorption of amino acids was best in those experiments where both inorganic phosphate and ATP were added to the mixture of amino acids introduced into the intestine. In these

* As in original – Publisher.

cases, the presence of labile phosphorus compounds of amino acids was observed in the intestinal wall.

The method for the determination of the labile phosphorus compounds of amino acids was to determine the fraction of labile phosphorus after the precipitation of the inorganic phosphate by a magnesia mixture (Belitser [11], Reiner [12], Ivanov and Severin [13]).

TABLE 1

The Influence of Inorganic Phosphate and Adenosinetriphosphoric Acid on the Absorption of Amino Acids in the Intestine

(The figures were obtained 30 minutes after the introduction of the mixture)

Rat groups	Nitrogen (mg)			Phosphorus (mg)					
	Introduced	Remained in the intestinal contents	Absorbed	Inorganic		Labile		Acid-soluble	
				A	Б	A	Б	A	Б
I	21.2	21.5	0	0.41	3.5	Traces		1.21	4.96
II	21.2	18.0	3.2	6.93	2.08	0	0	6.4	4.96
III	27.9	17.5	10.4	2.25	1.56	0.54	0.78	2.52	2.34

Note: a) in the intestinal contents; B) in the intestinal wall.

An even greater variation in the amounts of adsorbed amino acids was observed in experiments with a 60 minute interval. In the absence of phosphorus, 22% of the amino acid nitrogen introduced into the intestine was absorbed, while 47% was absorbed in the presence of phosphate alone, and 60% was absorbed in the presence of phosphate and ATP (Fig. 1).

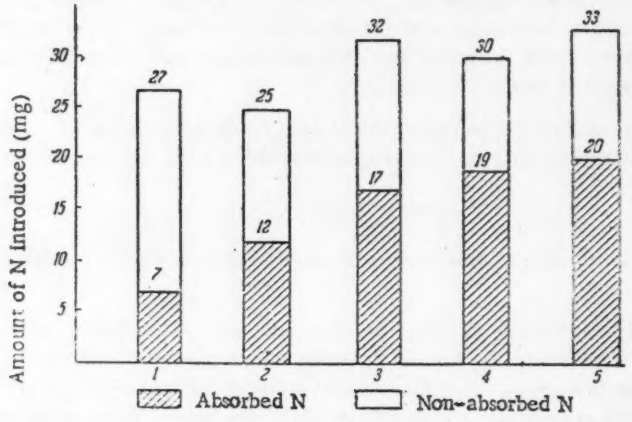

Fig. 1. Influence of inorganic phosphate and adenosinetriphosphoric acid on the absorption of amino acid nitrogen. The figures were obtained 60 minutes after the introduction of the mixture of amino acids:

1) Mixture I; 2) mixture II; 3) mixture I + 36 mg of inorganic phosphate + 25 mg of ATP; 4) mixture I + 30 mg of inorganic phosphate + 125 mg of ATP; 5) mixture I + 80 mg of inorganic phosphate + 250 mg of ATP.

The above experiments lent weight to our assumption of the role of phosphorylation in the process of amino acid absorption by demonstrating that the addition of phosphate and ATP increases the rate of amino acid absorption and leads to the formation of labile phosphorus compounds of amino acids in the intestinal wall. As a result of these experiments, as well as those of Wilson and Wiseman [14, 15], which demonstrated that the active absorption of methionine occurs only under aerobic conditions, we became interested in the conditions under which the synthesis of labile phosphorus compounds occurs in the intestine.

In order to elucidate this problem, we planned subsequent in vitro experiments, which were performed in the following manner. A mixture of amino acids dissolved in phosphate buffer, pH 7.4, to which a homogenate or extract of intestine and ATP were added, was incubated in a thermostat at 37° for 1 hour under aerobic and anaerobic conditions; the absorption of O_2 was measured. At the end of incubation, the mixture was precipitated on ice by trichloroacetic acid (final concentration of 2.5%). The residue was separated by centrifugation and the various fractions of phosphorus were determined in the solution. The labile phosphorus was determined after precipitation of the inorganic phosphorus by a magnesia mixture.

It was established that under anaerobic conditions of synthesis no labile phosphorus derivatives were formed. Under aerobic conditions, we observed the formation of significant amounts of labile phosphorus due to the demineralization of inorganic phosphate. At the same time, a significant absorption of oxygen occurred (Table 2). The amount of absorbed oxygen was the same at pH 6.5 and 7.4, while in an alkaline media of pH 9 it was significantly lower; the formation of labile phosphorus was stopped in an acid media of pH 6.5 as well as in an alkaline media of pH 9.0 and was most intensive at pH 7.3-7.4.

TABLE 2

Experiments on Oxidative Phosphorylation of Amino Acids, in the Presence of ATP, in Homogenates and Extracts of Intestine
(There were 10-20 mg of ATP at 25 mg of amino acids present, per sample)

O_2 absorption and formation of labile P	0	15 min.	30 min.	60 min.	NaF 30 min.	NaF 60 min.	pH	Source of enzyme	Added
1	—	30	80.0	160.2	80	124	7.3	Homogenate of intestine	
2	0.1	0.35	0.3	0.4	0.52	0.7			
1	—	36.4	72.2	140	62	123	7.3	Extract of intestine	
2	0.16	0.31	0.4	0.44	0.48	0.4			
1	—	0	14.8	44	0	24.5	8.4	Same	
2	0	0	0	0	0	0			
1	—	46	93.3	134	80	120	6.5	Homogenate of intestine	Succinic acid
2	0	0	0	0	0	0			
1	—	70	84.8	169.4	84	149	7.4	"	"
2	0.09	0.15	0.11	0.17	0.08	0.12			
1	—	6	29.7	55	25	24.5	9.1	"	"
2	0	0	0	0	0.08	0.07			

Note: 1) O_2 absorbed (1); 2) mg of labile P formed.

Having thus demonstrated the formation of phosphorylated amino acids in the intestinal wall, we now found it necessary to demonstrate more conclusively the relationship between the formation of labile phosphorus compounds of amino acids and the absorption of amino acids in the intestinal wall.

In order to demonstrate this relationship it was important to obtain substances which would encourage or inhibit the process of oxidative phosphorylation. It is known [16-20] that 2,4-dinitrophenol is a specific inhibitor of oxidative phosphorylation; as for agents enhancing phosphorylation — according to the data of

Belitser succinic acid and NaF increase the use of phosphate, and thereby encourage phosphorylation. This led us to study the influence of the above-mentioned substances on the processes of amino acid phosphorylation. However, it was found that sodium fluoride has no influence on this process, and that succinic acid even slows it down. Therefore, we did not use the above-mentioned agents to clarify the relationship between the processes of phosphorylation and amino acid absorption, but were compelled to restrict ourselves to experiments with dinitrophenol.

These experiments were preceded by determination of the period of most intensive absorption of amino acids and of the formation of labile phosphorus in the intestinal wall during the process of food digestion. For this we performed experiments on 60 rats which received raw meat containing a sufficient amount of phosphate after a 24 hour fast.

TABLE 3

Passage of Food, Absorption of Nitrogen and Content of Labile Phosphorus in the Intestinal Wall at Various Time Intervals During the Feeding of Meat to Rats

Order number	Time lapsed after feeding (hours)	1	2	3	4	5	6	7	8	9
1	Amt. N received with food, mg	270	335	350	350	356	350	353	356	356
2	Amt. N in stomach contents, mg	250	270	265	185	156	105	83	56	56
3	Amt. food passed into intestine, mg	1	1.7	2.9	4.7	5.8	6.4	7.5	8.0	8.4
4	Total amt. N passed into intesting, mg	20	65	90	165	200	245	270	300	300
4a	Amt. N passed into intestine after one hour, mg		45	25	75	35	45	25	30	0
5	Amt. N in intestinal contents, mg	10	11	9	10	10	8	10	7	7
6	Total amt. N absorbed in intestine, mg	10	54	81	155	190	237	260	293	293
6a	Amt. N absorbed after one hour, mg		44	27	74	35	47	23	33	0
7	Amt. N in intestinal wall per 1 g of tissue, mg	17	17	18	19	18	16	15	14	14
8	Amt. of inorganic P in intestine, mg	2.55	2.33	2.5	2.2	2.2	2.5	2.3	1.72	1.7
9	Amt. of readily hydrolyzable P in intestine, mg	0.29	0.42	0.6	0.5	0.5	0.25	0.2	0.2	0.2
10	Amt. of labile P in the intestine	0	0	0.35	0.8	0.49	0.34	0.95	0	0

The rats were separated into 10 groups of 6 rats each. These groups of rats were killed and examined at various intervals of time (from 1 to 10 hours) after feeding. The amount of food taken in and its content of nitrogen were taken into consideration. The stomach and intestines were removed from the rats and the nitrogen and phosphorus fractions were determined in their contents, while only the phosphorus fractions were determined in the intestinal wall. The amount of absorbed nitrogen was expressed as the difference between the nitrogen content of the food and the amount present in the contents of the stomach and intestine. These experiments showed that the most intensive passage of food from the stomach, as well as the most intensive amino acid absorption and formation of labile phosphorus, occurred 4 hours after feeding (Table 3, lines 3, 4, 4a, 6, 6a). The maximum passage of food into the intestine was accompanied by a decrease in the amount of inorganic phosphate (Table 3, line 8) and an increase in the fraction of labile phosphorus in the intestinal wall (Table 3, line 10).

It is important to stress that the periods of maximum amino acid absorption and of the most intensive formation of labile phosphorus, coincided. The absorption and nitrogen occurs relatively fast. While 165-200 mg of nitrogen had passed from the stomach into the intestine 4-5 hours after feeding, only 9-11 mg of nitrogen were recovered in the intestinal content, at this time (Table 3, line 5)*. At approximately the same time – the third to fifth hour (Table 3, line 7) the amount of nitrogen in the intestinal wall increased. Later on, it dropped to a value lower than the initial.

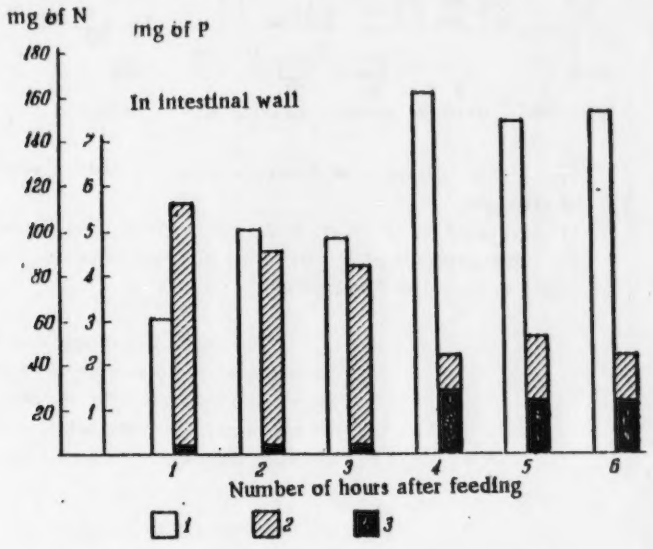

Fig. 2. Relationship between the passage of food from the stomach into the intestine, the absorption of nitrogen and the formation of a fraction of labile phosphorus compounds at the expense of inorganic phosphate:
1) Nitrogen absorbed (mg); 2) inorganic phosphorus (mg); 3) labile phosphorus (mg).

The same is clear from Figure 2 upon observation of individual experiments. The maximum passage of food from the stomach and the nitrogen absorption occur also 4 hours after the feeding of meat. The diagram clearly shows the relationship between the passage of nitrogen, the formation of labile phosphorus, and the disappearance of inorganic phosphorus.

Having thus established that the most intensive amino acid absorption occurs 4 hours after feeding, we followed with experiments using dinitrophenol.

In order to determine the relationship between the processes of phosphorylation and amino acid absorption we performed experiments on 20 rats. Inorganic phosphate and ATP were added to their basic food ration. The composition of the ration was as follows: starch 11 g, fat (sunflower oil) 1.5 g, egg albumin 20 g, phosphorus-free salt mixture 0.6 g, vitamins A and D, 1 drop of each, inorganic phosphate 400 mg, and ATP 100 mg. The animals were separated into 3 groups of which one was the control group, the second received 10 mg, and the third 50 mg, of dinitrophenol per day. The rats were kept in changeable cages and the content of nitrogen and phosphorus in the daily food ration, the urine and the feces was determined. The experiment lasted for 5 days. The experiments showed that the protein was 93% assimilated in the control group, 73% in the second group and 37.5% in the third group (Fig. 3).

* We attempted to slow down the absorption by producing amytal sleep, and observed a significant decrease in the rate of passage of the food from the stomach upon feeding of meat. Even by the 4th hour, an insignificant amount of the stomach contents had passed into the intestines.

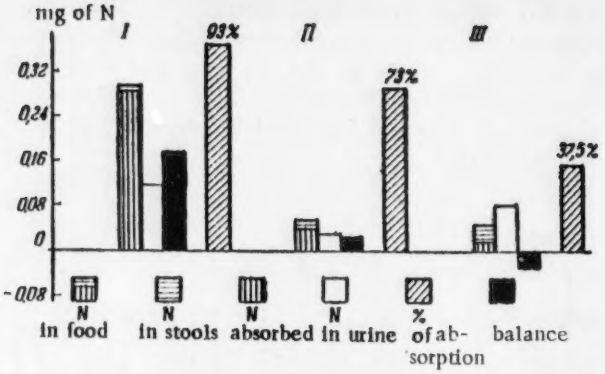

Fig. 3. The influence of dinitrophenol on the assimilation of nitrogen:
I) Average of 10; II) 10 mg of 2,4-dinitrophenol was added to the ration (average of 5); III) 50 mg of 2,4-dinitrophenol was added to the ration (average of 5).

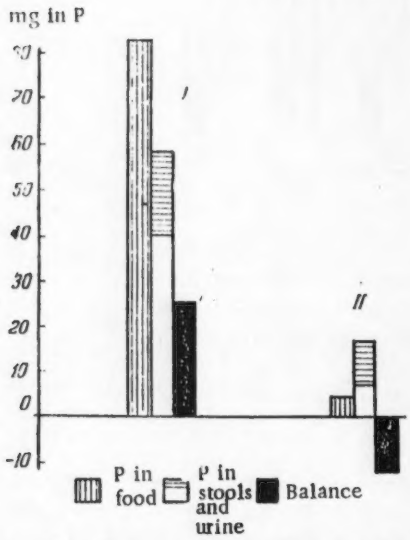

Fig. 4. The influence of dinitrophenol on the assimilation of phosphorus (mg):
I) 400 mg of phosphates and 100 mg of ATP were added to the food ration (average of 10), II) phosphate, ATP and 50 mg of dinitrophenol were added to the food ration (average of 5).

The balance of nitrogen and phosphorus (Fig. 4) in rats receiving dinitrophenol was negative. A significant loss of weight was observed in these animals. As seen from Fig. 4 the balance of phosphorus was negative only when dinitrophenol was added to the food ration.

At the end of the studies on assimilation the rats were killed and the amount of nitrogen and labile phosphorus was determined in the intestinal contents and in the intestinal wall. An increase of the amount of nitrogen in the intestinal wall (154 mg in the whole intestine instead of 138 mg without the addition of phosphate and ATP), as well as of the amount of labile phosphorus (an average of 1.6 mg instead of 0.16 mg) was observed 4 hours after feeding of rats which received additional phosphate (400 mg) and ATP (100 mg) (see Table 4). Upon addition of 50 mg of dinitrophenol to the daily food ration the amount of nitrogen present in the intestinal wall decreased (103 mg instead of 153 mg). The labile phosphorus was totally absent from the intestinal wall.

Therefore, the relationship between the intensity of the processes of phosphorylation and the intensity of amino acid absorption in the intestines was clearly established in this series of experiments.

Our results allowed us to assume that the poor assimilation of proteins of some food products may be due to their low content or total lack of free phosphate and ATP. For instance, bran which does not contain ATP and which has non-assimilable phytin phosphorus (the latter, as demonstrated by the investigation of Vasilyeva [21], is not absorbed in the intestine) belongs to such products.

As known, bran proteins are characterized by poor assimilation. If our contention that the poor assimilation of proteins may be due to the low content of assimilable phosphorus and ATP in the food products is correct, we can then assume that the addition of phosphate and ATP to a food consisting basically of bran will raise the assimilation of bran proteins.

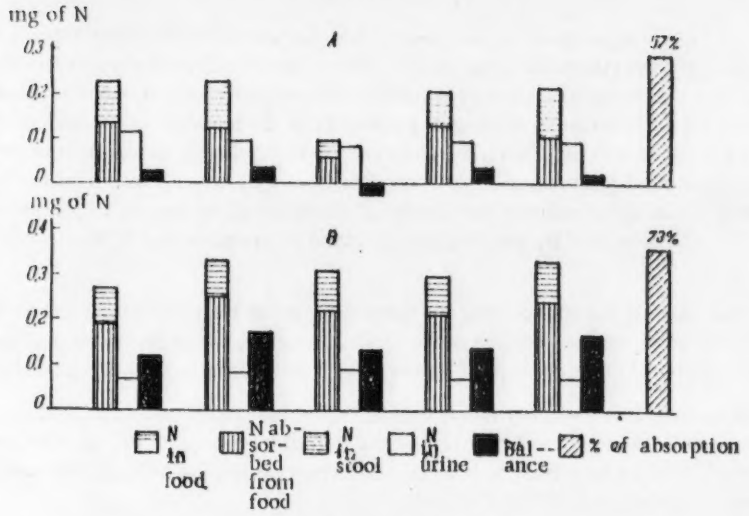

Fig. 5. Absorption of nitrogen from bran:
A) without the addition of phosphate and ATP; B) with the addition of 100 mg of ATP and 200 mg of phosphate to the food ration.

TABLE 4

Content of Nitrogen and Labile Phosphorus in the Intestinal Wall 4 Hours after Feeding

Investigated factors	Without addition of phosphate and ATP to the food ration	With addition of ATP (100 mg) and phosphate (400 mg)	35 mg dinitrophenol added to food ration, in addition to phosphate and ATP
N in intestinal wall, mg	138	154	103
Weight of intestine	7.0	7.6	5.8
N absorbed during 4 hours, mg	69	101-150	5
N in intestinal contents, mg	14	8	6
P in the intestinal wall, mg	1.8	2.65	1.7
Labile P in the intestinal wall, mg	0.16	1.6	0
Total P in the intestinal wall, mg	18.5	21.8	15
Inorganic P in intestinal contents	Traces	0.6	0.45
Labile P in intestinal contents	0	0.4	0
Variations in the weight of rats	−2 +3 g	+7 +20 g	−50 g

In order to determine the accuracy of our assumption we performed experiments on 10 rats. The diet of these rats consisted of starch, fat, a phosphorus-free salt mixture, and contained wheat bran as the source of protein. The rats were separated into 2 groups of 5 rats each. The rats of the second group received in addition to their basic diet, 400 mg of phosphorus and 100 mg of ATP. The rats of the first group did not receive these additions. After a 5 day feeding, the rats were placed into changeable cages for 4 days. The nitrogen content was determined in the 4-day food ration, in the urine and feces. The latter were separated by carmine.

The results are presented on Figure 5 from which it is seen that, under the influence of phosphate and ATP, the assimilation of bran protein is increased by 20% in comparison with the control.

One may notice that the nitrogen balance in rats which received phosphate and ATP was clearly positive and that at the same time these rats increased in weight. In those rats which did not receive phosphate and ATP the nitrogen balance was usually almost equal to 0 (in 2 cases it was negative). At the same time, these rats either lost weight or their weight remained unchanged.

DISCUSSION

On the basis of our experimental data we may assume that the absorption of amino acids is an active process which is coupled with the phosphorylation of amino acids. This is supported by the above-described experimental data, which have shown the possibility of a biological synthesis of phosphorylated amino acids in the intestinal wall under aerobic conditions; labile phosphorus compounds are formed in the intestine at the time of the intensive passage of food from the stomach into the intestine and during the absorption of the products of protein digestion; a discontinuance of the formation of labile phosphorus in the intestinal wall, as well as a significant decrease in the assimilation of protein nitrogen, occur upon the dinitrophenol inhibition of the process of phosphorylation. The assimilation of protein may be increased by the addition of inorganic phosphate and ATP to poorly assimilable proteins.

We cannot maintain that all the amino acids are phosphorylated to the same extent and at the same rate. It is possible that some amino acids are not phosphorylated at all. However, the experiments described above demonstrate that the absorption of the main bulk of amino acids is coupled with their phosphorylation.

Our data also demonstrate that the conditions which influence the process of amino acid phosphorylation should be considered as one of the factors which influence the assimilation of proteins. In those cases when a poor assimilation of proteins is due to an inhibition or absence of phosphorylation the assimilation may be increased by the addition of inorganic phosphate and ATP to the food.

SUMMARY

The intestinal absorption of the main bulk of amino acids is coupled with their phosphorylation and is inhibited upon the disruption of this process. The intensity of the process of amino acid phosphorylation in the intestine should be considered as one of the factors influencing the assimilation of proteins. In those cases when a poor assimilation of proteins is due to the absence of the conditions necessary for the phosphorylation of amino acids, the addition of phosphate and ATP to the food may increase the assimilation of food proteins.

In conclusion I wish to express my gratitude to Dr. Med. Sci. Z. D. Frunin and Professor A. E. Sharpenak for their advice during the course of this work.

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ON THE FORMATION OF ALANINE, GLUTAMIC AND ASPARTIC ACIDS FROM FROM KETO ACIDS AND AMMONIUM SALTS IN SLICES OF RAT LIVER

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The problem of the formation of amino acids from keto acids and ammonium salts is still confined to the question of whether or not the direct amination if pyruvic acid in liver and kidney tissues with the immediate formation of alanine is possible. After Neber [1] observed an intensive formation of amino nitrogen in the presence of pyruvic and ammonium salts in liver and kidney slices, we demonstrated [2] that this process occurs with almost the same intensity in the presence of ketoglutaric and oxalacetic acids as substrates. In this case, we observed a preferential formation of dicarbonic amino acids, while predominantly alanine formed in the presence of pyruvic acid as substrate. This led us to assume that upon incubation of liver and kidney slices with keto acids and ammonium salts a direct amination of pyruvic acid, as well as ketoglutaric and oxalacetic acids, occurs.

However, a completely different mechanism of alanine and pyruvic acid synthesis was advanced in the work of Kritsman [3], which was published somewhat later. On the basis of her observations that this process occurs only when the slices are incubated in bicarbonate buffer Kritsman came to the conclusion that no direct amination of pyruvic acid occurred; according to her, in a media containing carbon dioxide, pyruvic acid combines with the latter and forms oxaloacetic acid, which acts as the direct acceptor of ammonia. Alanine is then formed from the aspartic acid which is obtained by transamination of the excess of pyruvic acid through the latter scheme. However, the data of Kritsman on absence of synthesis of amino acid from pyruvic acid in a media free of carbon dioxide was not confirmed by our experiments [4] nor by the investigations of Wiss [5] and Sinitsyna [6]. In a subsequent work, Kritsman and Melik-Sarkisyan [7] refuted their own explanations of the synthesis mechanism in kidney tissues but continued to hold their views on the formation of amino acids in liver tissue. To substantiate their contentions, they provided data showing that during the first 30 minutes of incubation of liver slices with pyruvic acid the amount of aspartic acid formed was 2-3 times larger than the amount of alanine. These data were obtained by Darling's quantitative method of amino acid separation and determination. In later work, however, Sinitsyna found by paper chromatography that upon a 30 minute incubation of liver slices with pyruvic acid and ammonium salts only alanine and glutamic acid were formed, while aspartic acid formed in relatively very small quantities.

On the basis of her investigations on livers of animals deficient in vitamin B_6, in which the transamination processes were significantly disturbed, and on the basis of the results of the experiments of Berezov [8], who studied the influence of vitamin B_6 deficiency on the formation of l-amino acid isomers from d-isomers, Sinitsyna came to the conclusion that the main, if not the only, ammonia acceptor in liver and kidney tissues is ketoglutaric acid. Thus, according to Sinitsyna, the immediate product of amination is glutamic acid, which forms other amino acids via transamination. Sinitsyna explains the synthesis of amino acids in the presence of pyruvic acid as the substrate by the formation of ketoglutaric acid from pyruvic acid in the tricarboxylic acid cycle.

As can be seen from the above, the conclusions on the modes of alanine formation from pyruvic acid and ammonia were based for the most part on the amounts of one or the other amino acid which form upon various periods of incubation. Due to the presence of sharp divergencies in the existing information we decided to investigate this problem once more by applying one of the most accurate contemporary methods, namely quantitative paper chromatography for the determination of amino acids.

EXPERIMENTAL

The experiments were performed on liver slices from adult white rats. The slices were incubated under aerobic and anaerobic conditions at 37°, for various periods of time with pyruvic, ketoglutaric or oxaloacetic

acids and ammonium carbonate. The final concentration of the added keto acids was 0.05 M, and that of ammonium carbonate 0.02 M. Slices incubated in a media without the addition of substrates were used as controls.

After the end of incubation the proteins were precipitated by trichloroacetic acid, which was then removed from the filtrates by ether extraction. In one part of the experiments the amino acids formed were qualitatively determined by paper chromatography (advancing phase, phenol saturated with water). In another series of experiments we quantitatively determined the amino acids along with the keto acid which formed. This enabled us to estimate the balance of amino acid formation. For the quantitative determination of amino acids we used the ninhydrin method of Troll and Cannon as modified by Paskhina [9], which was devised by her for the determination of amino acids after their chromatographic separation on paper. The pyruvic and ketoglutaric acids were determined in a part of the filtrates from which the proteins were removed by a sodium tungstate solution according to Cavallini and Frontali [10]. The keto acids were isolated as hydrazones and separated by paper chromatography from butanol-ethanol-water mixture (40 : 10 : 50). After the elution of hydrazones from the paper their amounts were determined spectrographically (SF-4 spectrophotometor at 510 mμ).

RESULTS

In the first series of experiments we determined the amino acids which formed upon incubation of liver slices with pyruvic, ketoglutaric or oxaloacetic acids for various periods of time. The results of these experiments are shown on the chromatograms (Figs. 1-3).

On the Fig. 1 chromatogram (with addition of pyruvic acid) it may be observed, that after only 30 minutes incubation, the alanine spot is clearly apparent, while those of glutamic and aspartic acids are much less so. As the time of incubation increases the intensity of the alanine spot also significantly increases and the intensity of the glutamic and aspartic acid spots also increases somewhat. In the case when ketoglutaric acid forms the amination substrate (chromatogram, Fig. 2) a preferential formation of glutamic acid occurs during the first 30 minutes. The amount of alanine formed during this period is relatively small and increases only little upon further incubation. A somewhat different picture was obtained upon incubation of slices with oxaloacetic acid. As seen from the Fig. 3 chromatogram, a large spot of alanine is obtained along with a clearly apparent spot of aspartic acid, after 30 minutes. Upon further incubation, the spots of both aspartic acid and alanine are increased. Inasmuch as oxaloacetic acid is to a large extent spontaneously decarboxylated with the formation of pyruvic acid upon incubation, it is quite probable that the amination of the latter determines the formation of definite alanine spots on the corresponding chromatograms.

Our data obtained in this series of experiments are in contradiction to those obtained by Kritsman as well as Sinitsyna under the same conditions.

Kritsman shows that aspartic acid is predominantly formed during the first 30 minutes of incubation of the liver slices with pyruvic acid, and that its amount is 2-3 times larger than the amounts of alanine and glutamic acid formed during the same period of time. In the work of Sinitsyna, to the extent that it can be estimated by the given chromatograms, approximatively equal amounts of alanine and glutamic acids and a significantly smaller amount of aspartic acid are formed upon the incubation of liver slices for 30 minutes with pyruvic acid.

Due to the sharp divergencies between our data and the results obtained by the latter authors, we performed, in the next series of experiments, a quantitative analysis of the formed amino acids.

Since the salts which enter into the composition of the Ringer solution interfers with the quantitative determination of amino acids by the method of Paskhina, the incubation of liver slices with keto acid and ammonium carbonate was performed, in this series of experiments, in a 0.9% NaCl solution. It was determined in previous experiments that the substitution of a 0.9% NaCl solution for the Ringer solution did not influence the amount of amino acid which forms upon incubation.

We determined the total amount of amino acids formed by the Van-Slyke method as well as by the decrease of pyruvic acid used as the substrate of amination during the process of incubation, and the formation of ketoglutaric acid.

As can be seen from Table 1, alanine synthesis predominated in all of the experiments on the incubation of liver slices with pyruvic acid, independently of the duration of incubation. The amount of alanine formed during the first 30 minutes was almost 20 times the amount of aspartic and glutamic acids. The same relationships remained upon incubation for 2 and 3 hours. The total increase of amino nitrogen for the time of incubation

TABLE 1

Increase of Amino Acids upon Incubation of Liver Slices with Ammonia and Pyruvic Acid under Aerobic Conditions

500 mg of slices; 0.9% solution of NaCl; 95% O_2 + 5% CO_2 (or N_2); final concentration of pyruvic acid – 0.05 M; of ammonium carbonate – 0.02 M; volume of samples – 7.5 ml.

Expt. no.	Duration of incubation	Increase of total amino nitrogen according to Van Slyke (μM/g of tissue)	Increase in amino acid (μM/g tissue)			
			Alanine	Aspartic acid	Glutamic acid	Total
1	30 min.	56	57.2	3.0	5.7	65.9
	2 hr.	136	128.6	5.2	12.0	145.8
	3 hr.	200	157	8.2	17.2	182.6
2	30 min.	52.8	27	3.0	1.4	31.6
	2 hr.	157.2	122.8	7.2	9.2	139.2
	3 hr.	181.4	147	9.4	12.0	168.6
3*	30 min.	74	78.6	—	12.8	91.4
	2 hr.	222	204	—	28.6	232.8
	3 hr.	300	271	—	51.4	322.8

* In Experiment 3, aspartic and glutamic acids were determined simultaneously.

TABLE 2

Increase in Amino Acids upon Incubation of Liver Slices with Ammonia and Pyruvic Acid under Anaerobic Conditions
(see Table 1 for conditions)

Expt. No.	Duration of incubation	Increase of total amino nitrogen according to Van Slyke (μM/g tissue)	Increase of amino acids (μM/g of tissue			
			Alanine	Aspartic acid	Glutamic acid	Total
4	30 min.	52.8	62.8	0	0	62.8
	2 hrs.	142	121.4	0	0	121.4
	3 hrs.	167	147	0	0	147
5	30 min.	82	77	0	0	77
	2 hrs.	139	124	0	0	124

determined by the Van Slyke method is in good agreement with the sum of the increase of individual amino acids determined chromatographically, which confirms the accuracy of our determinations.

In one of the experiments of this series we significantly decreased the concentration of pyruvic acid in the incubating mixture in order to make it more like that of physiological concentrations. Instead of 400 μM per sample we added only 80 μM per sample in this experiment; however, the results of this experiment were the same as those obtained in experiments with high concentrations of pyruvic acid.

In Table 2 and on the Fig. 4 chromatogram the data obtained in two experiments performed under anaerobic conditions are given. The only distinguishing feature of the results of these experiments was that under anaerobic conditions no accumulation of aspartic and glutamic acids occurs at all, and the only product of the reaction is alanine.

Fig. 1. The formation from pyruvic acid and ammonia of amino acids in slices of liver of a normal rat, upon incubation for: 1) 30 minutes; 2) 2 hours; 3) 3 hours. Substrates in samples 1, 2 and 3) Na pyruvate and ammonium carbonate; 4) pyruvate; 5) ammonium carbonate; 6) Ringer-bicarbonate; 7) spots: aspartic acid, glutamic acid and alanine, 6 μg of each. The amounts of chromatographed filtrates corresponded to 1.6 mg of the weight of the initial tissue.

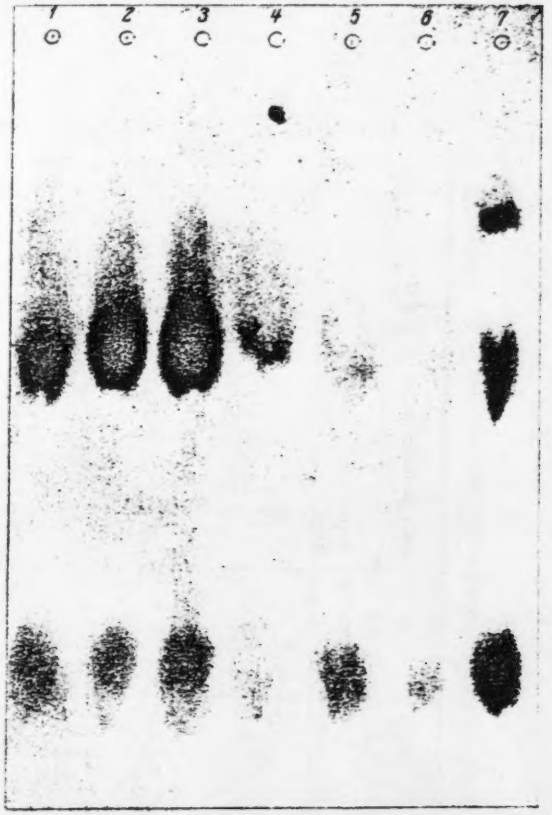

Fig. 2 The formation from ketoglutaric acid and ammonia of amino acids in slices of a liver of a normal rat upon incubation for:
1) 30 minutes; 2) 2 hours; 3) 3 hours. Substrates in samples 1,2 and 3) ketoglutaric acid and ammonium carbonate; 4) ketoglutaric acid; 5) ammonium carbonate; 6) Ringer-bicarbonate.

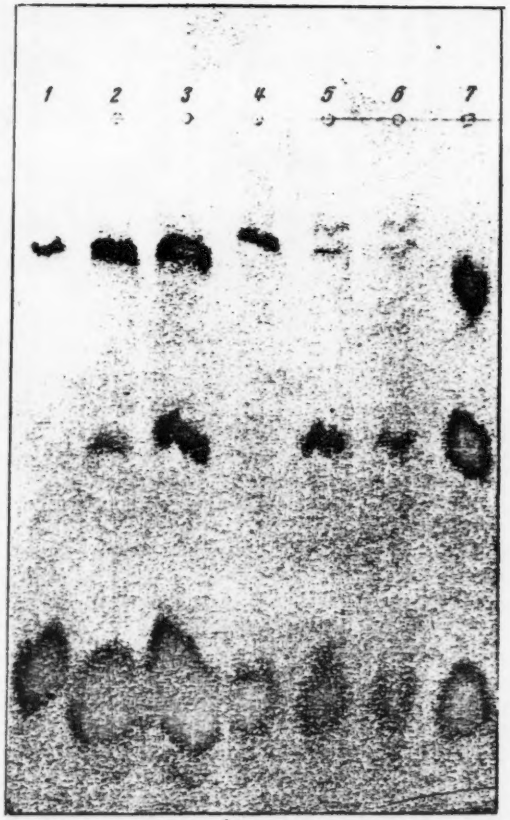

Fig. 3. The formation from oxaloacetic acid and ammonia of amino acids in slices of liver of a normal rat upon incubation for:
1) 30 minutes; 2) 2 hours; 3) 3 hours. Substrates in samples 1, 2 and 3) oxaloacetic acid and ammonium carbonate; 4) oxaloacetic acid; 5) ammonium carbonate; 6) Ringer-bicarbonate.

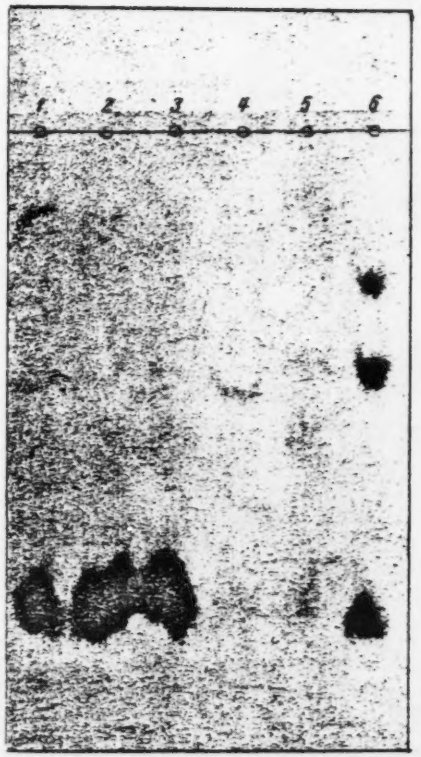

Fig. 4. The formation from pyruvic acid and ammonia of amino acids in slices of rat liver under anaerobic conditions and upon incubation for:
1) 30 minutes; 2) 2 hours; 3) 3 hours. Substrates of stages 1, 2 and 3) pyruvate and ammonium carbonate; 4) pyruvate, 5) ammonium carbonate; 6) Ringer-bicarbonate.

TABLE 3

Increase in Amino Acids upon Incubation of Liver Slices with Ammonia, Pyruvic and Ketoglutaric Acids Simultaneously

400 mg of slices; NaCl: 0.9% 95% O_2 + 5% CO_2; pyruvic and ketoglutaric acids – 0.05 M (each); ammonium carbonate – 0.02 M; volume of samples – 6 ml

Added substrates	Duration of incubation	Increase in amino acids (μM/g of tissue)			
		Alanine	Glutamic acid	Aspartic acid	Total
Pyruvic	30 min.	18.5	2.0	4.0	24.5
Ketoglutaric		3.7	24.5	0	28.2
Pyruvic + ketoglutaric		25.7	32	7.7	65.4
Pyruvic	2 hrs.	83.2	6.5	13	102.7
Ketoglutaric		8.7	66.7	5.7	81.1
Pyruvic + ketoglutaric		87.7	56.7	25.2	169.6

The results of the experiments in which the slices were incubated simultaneously with pyruvic and ketoglutaric acids are presented in Table 3 along with the results of the experiments on samples to which only pyruvic or ketoglutaric acids were added (the final concentration of each keto acid in all samples was 0.05 M). It is apparent from this table that the amounts of alanine and glutamic acid synthesized in the samples to which both substrates were added at the same time are approximately the same as those that were synthesized in samples to which the corresponding substrates were added separately. This indicates that the amination process of each keto acid occurs independently from the amination of the other keto acid. This gives a basis for the assumption that their amination is catalyzed by separate enzyme systems.

As indicated above, we determined in a number of experiments the variations in the amount of keto acids present in the incubating mixture along with determinations of the amount of amino acids formed. This allowed us to estimate to a certain extent the amount of pyruvic acid used for the synthesis of amino acids and the amount of ketoglutaric acid formed from it in the tricarboxylic acid cycle. The corresponding results are given in Table 4.

As can be seen from Table 4, the results of all experiments agree quite well. During the first 30 minutes of incubation 78 to 138 μM of pyruvic acid (per 1 g of tissue), or from 10 to 18% of the added amount, is consumed. Upon further incubation the consumption occurs at approximately the same rate. When these values are compared to the amounts of amino acids formed (Tables 1 and 2) it becomes apparent that approximately 50% of the pyruvic acid which is consumed during the first 30 minutes of incubation is used for the synthesis of alanine. Upon further incubation, the amount of pyruvic acid consumed decreased somewhat, but did not fall below 30%.

The amount of accumulating ketoglutaric acid did not as a rule surpass 15-16 μM, i.e., was not greater than 2% of the added pyruvic acid. The conditions of our experiments do not allow us to decide whether the indicated amount of ketoglutaric acid is formed from pyruvic acid in the tricarboxylic acid cycle or whether it accumulates as a result of the transamination of glutamic acid which is present in the tissues along with pyruvic acid.

DISCUSSION

The above data demonstrate beyond any doubt that the corresponding amino acids are predominantly formed from pyruvic, ketoglutaric and oxaloacetic acid upon incubation for 30 minutes as well as for 2 hours. Upon incubation of liver slices with pyruvic acid and ammonium salts in various buffers we were unable to observe any significant formation of glutamic or aspartic acids. Therefore, these data support our preliminary assumption that each of the investigated keto acids may act as the substrate for direct amination.

TABLE 4

Variations in the Content of Pyruvic and Ketoglutaric Acids upon Incubation of Liver Slices with Ammonia and Pyruvic Acid
(see Table 1 for conditions)

Expt. No.	Duration of incubation	Amount of pyruvic acid added (μM/g of tissue)	Amount of pyruvic acid recovered (μM/g of tissue)	Amount of ketoglutaric acid recovered (μM/g of tissue)
1	0	748	—	—/
	30 min.		608	7
	2 hrs.		440	15.6
	3 hrs.		414	11.8
2	0	748	—	—
	30 min.		668	2.4
	2 hrs.		440	5.6
	3 hrs.		301	10
3	0	748	—	—
	30 min.		662.8	18.4
	2 hrs.		346	38.2
	3 hrs.		94	33.8
4*	0	748	—	—
	30 min.		662.8	11
	2 hrs.		474	21.4
	3 hrs.		393	16.6
5*	0	748	—	—
	30 min.		631	9.2
	2 hrs.		458	13.6

* In Experiments 4 and 5 the slices were incubatic under anaerobic conditions.

Those experiments in which pyruvic and ketoglutaric acids acted together as the substrates for amination are especially convincing in this respect. Since the molar concentrations of pyruvic and ketoglutaric acids were identical in these experiments, the subsequent process of transamination between the forming amino acids and the excess of keto acids may have occurred only to an insignificant extent, and the amount of each amino acid formed should have basically reflected the intensity of its formation from the corresponding keto acid by way of direct amination. The results of these experiments have in fact shown that the amounts of alanine and glutamic acid formed were approximately the same. If we start from the assumption that ketoglutaric acid is the only substrate for amination, then the formation of alanine under the given conditions (the presence of an excess of ketoglutaric acid in the media) should not have occurred in any noticeable amounts. Therefore the formation in these experiments of the same amounts of alanine as in experiments in which the slices were incubated only with pyruvic acid is a serious objection to the assumption that ketoglutaric acid is the only substrate for amination.

It is rather difficult at the present time to explain with any certainty the results of our experiments on the amination of pyruvic acid under anaerobic conditions. On the one hand, the formation in these experiments of alanine alone supports the assumption of a direct amination of pyruvic acid. On the other hand, however, one should not exclude the possibility that under anaerobic conditions some ketoglutaric acid may be formed from pyruvic acid. The formation of small amounts of this acid was in fact observed in our experiments (Table 4). However, we cannot yet say anything definite on its source.

SUMMARY

Upon the incubation of liver slices with pyruvic acid and ammonium salts under aerobic and anaerobic conditions for 30 minutes and 2 hours the amount of alanine produced was many times larger than the resulting amounts of glutamic and aspartic acids. On the contrary, upon incubation of the slices with ketoglutaric acid, mainly glutamic acid was formed.

Upon incubation of the liver slices with ketoglutaric and pyruvic acids simultaneously, the formation of alanine as well as of glutamic acid occurs. This indicates that in liver slices the formation of each amino acid from the corresponding keto acid occurs independently from each other.

The resulting amount of amino acid, upon incubation of liver slices with pyruvic acid, represents approximately 50% of the pyruvic acid which is consumed during the experiment.

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TRANSFORMATION OF A PHOTOCHEMICALLY ACTIVE FORM OF CHLOROPHYLL IN SUGAR BEET LEAVES

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It was found in our preceding experiments [1] that chlorophyll, newly formed from protochlorophyll is in a photochemically active form which is destroyed extremely fast upon illumination with red light of high intensity. The main red maximum of this form of chlorophyll in its natural state is at 670 mμ. During the maturing process of etiolated seedlings and during their accumulation of chlorophyll, the absorption maximum gradually shifts toward the longer wavelengths to 678 mμ. At the same time there is a gradual increase in the resistance of the pigment to the action of light. However, at all stages of chlorophyll accumulation as well as in the "mature" plant some of the chlorophyll occurs in a photochemically labile form. The bleaching of this form upon illumination with red light is accompanied by a significant decrease in the fluorescence of the leaves and of the "green solutions". In accordance with the preliminary investigations of D. I. Ivanovsky the main mass of chlorophyll in the leaves is found in an aggregated, photochemically stable state [1, 2].

In the above mentioned experiments, we found some indications that the balance between the aggregated and the monomeric forms is determined by the physiological conditions of the plant.

The presence in plant leaves of a photochemically unstable, monomeric and fluorescing form of chlorophyll observed in our investigations is in accord with the experimental results of other authors. Thus, French and Young [3] observed a sharp decrease in the intensity of the red fluorescence upon excitation of the red seaweed Porphyra naiadum with light of high intensity during measurements of its fluorescence spectra. The differential fluorescence spectra, expressed as the difference between the spectra obtained at low and high intensity of the exciting light, is a curve with its maximum at 677 mμ corresponding to the fluorescence maximum of chlorophyll in its "monomeric" form. The authors give no explanation of this phenomenon. However, it is doubtless true that light of high intensity leads to the bleaching of the fluorescing form of chlorophyll. In fact measurements of the fluorescence spectra of young, partly green, and "mature" leaves in the work of French and Young, showed differences in the position of the main fluorescence maximum (in a dark-green leaf, at 685 mμ and in a light-green, young leaf, at 675 mμ). According to our data the fluorescence spectra of a leaf is determined mainly by the monomeric, active form of chlorophyll with its absorption maximum at 670 mμ. The shift in the position of the fluorescence maximum in a young leaf is insignificant due to the secondary absorption, while a large amount of chlorophyll in a dark-green leaf leads to a partial absorption of the short wavelength sector of the fluorescence maximum of the monomeric form which in turn leads to a noticeable shift of the maximum toward a region of longer wavelength (685 mμ). Of importance is the fact that the position of the second fluorescence maximum (at 720 mμ) is not shifted but retains its position in light-green and dark-green leaves [3]. Yocum and Blinks [4] investigating the quantum efficiency of photosynthesis in red algae also came to the conclusion that the active and inactive forms of chlorophyll were present in these organisms. It would be appropriate also to mention here the observations of Koski and Smith [5] who obtained a mutant of corn in which a small amount of protochlorophyll converted upon illumination into chlorophyll which quickly decomposed under the action of light. On the basis of our data there is no doubt that in these experiments the authors dealt with the formation of a monomeric, unstable form of chlorophyll from protochlorophyll.

In the present article we describe our search for the conditions which determine the amount of the active form of chlorophyll in plants, and those experiments in which we observed the reversible oxidation-reduction conversion of this form of chlorophyll in its native state. The basic results of this work were described earlier [6]. All of the properties and conversions of chlorophyll were investigated in colloidal or "green solutions" of the leaf substance prepared in water-glycerin media. The absorption maximum of chlorophyll in these "solutions" corresponds to the absorption of chlorophyll in leaves. The use of aqueous glycerin as the media allows the formation of translucent,

stable and viscous colloidal "solutions", permitting accurate spectrophotometric measurements and low temperature experiments. The addition of glycerin does not influence the position of the red absorption maximum of chlorophyll as compared to that of the aqueous "solution". Water-glycerin solutions exhibit the usual Hill reaction (with phenolindophenol). According to our previous investigations [7], this serves to verify the preservation of the natural state of chlorophyll. While we did not perform any morphological studies, there are, however, reasons to assume that the green solutions contain granules and structural elements of granules.

EXPERIMENTAL

The experiments were mainly performed on leaves of the sugar beet (variety "Uladovka" and "Verkhnyachka"). In the summer, the experiments were performed on sugar beet plants that were grown in the field in May-August (absorption maximum ay 675-678 mμ at pH 8.5). In the winter, the experiments were performed on beet leaves grown from roots in pots with soil under two different types of illumination. The plants growing close to the window had dark-green leaves with normal chlorophyll (red absorption maximum: 675-678 mμ at pH 8.5). The leaves grown in diffuse light, deep inside of the room, were of a pale-green color and had their absorption maximum at 672-673 mμ (at pH 7 and 8.5).

In order to prepare the green solution the leaves were minced in a mortar with 1/15 M phosphate buffer solution containing 1/50 M KCl. The buffer solution, pH 8.5, was a 1/15 M solution of Na_2HPO_4; NaOH was added to this buffer in order to obtain solutions of a more alkaline pH. The homogenate was strained through linen and centrifuged for 10 minutes at 3500 g. The obtained green solution was mixed with glycerin in the ratio of 4:6. All of these preparations were performed in a shaded room.

The green solution was diluted with the water-glycerin buffer solution (4:6) of the same pH up to an absorbance (A) of 0.6-0.7 at the red absorption maximum and a depth of solution of 1-1.2 cm. The accuracy of the determination of the absorption maximum is usually within the range of ± 1 mμ. The experiments were carried out in evacuated tubes or cylindrical cuvettes. For determination of the amount of the bleaching form of chlorophyll the tube with the green solution was alternately placed in the holder of the Beckman spectrophotometer and in a glass vessel where was circulating some water from a thermostat at a temperature of 20-22°; in experiments at a temperature of −40° the water was replaced by a mixture of carbon dioxide in acetone. The tube in the cylinder was illuminated by a 500 W cinematographic bulb with a condenser, through a red filter RG-2 (approximately 10^6 ergs/cm^2/sec.).

During the bleaching process we followed the decrease of A in the red absorption maximum. The measurements were made at the following times (after the beginning of illumination): 5, 10, 15, 25, 40, 70, 130, 310 seconds. The time-lapse between the end of illumination and the measurement was approximately 10 seconds.

The photochemical activity (the Hill reaction) of the green solutions was determined by the reduction of phenolindophenol and dichlorophenolindophenol [8]. In experiments involving phenolindophenol, the green solution and the dye solution were prepared in a buffer of pH 6.5 with the addition of 60% glycerin in such a way that A at the absorption maximum of the dye at 550 mμ would be equal to 1.0. In the case of dichlorophenolindophenol the solutions were prepared in a buffer of pH 7, 7.5 and 8.5 (A = 1 at 600 mμ). Determinations of the photochemical activity were performed in evacuated tubes with side-arms which allowed the addition of the dye (2.5 ml) from the side-arm to the green solution (2.5 ml) after evacuation, in order to avoid a premature reduction of the dye by the green solution in darkness. The absence of air is also necessary to prevent noticeable decomposition of the chlorophyll upon illumination. The illumination of the tube was carried out as described above at the following periods of time (from the beginning of illumination): 5, 10, 15, 20, 25 minutes. Each experiment had its "dark control", in which the variations of the concentration of the dye in the evacuated solution upon standing in darkness, were determined.

The changes of concentration of the dye characterizing the photochemical activity were measured by the decrease of A at the absorption maximum of the dye in the experiment under light, taking into account the dark control. In the presence of glycerin, the activity appeared somewhat lower when determined with phenolindophenol, while when determined with dichlorophenolindophenol the activity was somewhat higher in the presence of glycerin than in the experiment without glycerin. The latter may be explained by the fact that the reduction of dichlorophenolindophenol is determined not only by the Hill reaction but also by other processes as, for instance, the sensitization of chlorophyll and the reduction of dichlorophenolindophenol by glycerin. However, upon illumination of the solution of dichlorophenolindophenol in 60% glycerin, A, at the absorption maximum of the dye, at 600 mμ, did not change, i.e. no direct photoreduction of indophenol was observed.

The Kinetics of Chlorophyll Bleaching in Green Solutions

Upon illumination of the green solutions in the presence of atmospheric oxygen a fast decrease of A occurs at the absorption maximum of chlorophyll (Fig. 1). In the absence of air this phenomena is not observed. Therefore, the bleaching is a result of the oxidative decomposition of chlorophyll.

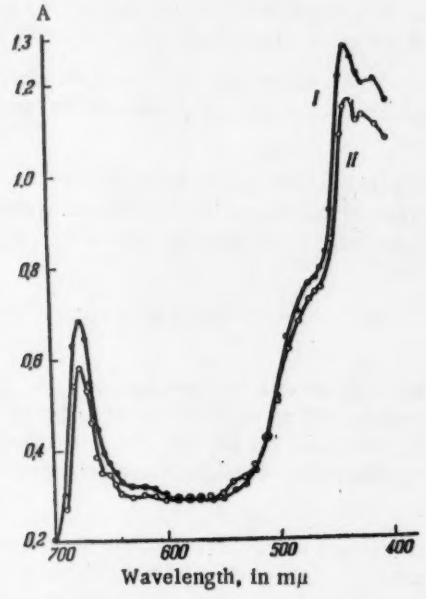

Fig. 1. Bleaching of chlorophyll in the green solutions from sugar beet leaves:
I) initial green solution, II) after 20 minutes illumination in the presence of air, through a red RG-2 filter.

The determination of A as a function of the duration of illumination gives a kinetic curve which describes the fast decrease of A during the first 5 seconds illumination; later on, the decrease of A occurs much more slowly (see Figs. 3, 5, and 9)*. Thus, for instance, in a typical experiment with green solutions of beet leaves 20% of the initial amount of chlorophyll present in the green solution is bleached during the first 5 seconds, 24% in 40 seconds, and 29% in 310 seconds. The form of the kinetic curve varies in green solutions prepared from leaves of plants grown under various physiological conditions.

In the green solutions of beet leaves grown in the winter close to the window, a significantly smaller percentage of chlorophyll is bleached than in "solutions" of leaves from plants grown in the field. After a lengthy period of cold, cloudy days there is almost no bleaching of the chlorophyll in the "solution" from leaves of winter beet plants either at pH 7 or at pH 8.5. Therefore, the kinetic curve testifies to the presence in the green solution of various forms of chlorophyll which bleach at various rates. The fast decrease of A at the absorption maximum of chlorophyll, at the beginning of the illumination of the green solution, is explained by the decomposition of the labile, monomeric form. The loss of fluorescence in the green solutions as a result of illumination testifies to the same fact (this may be observed through a RG-5 filter upon the production of fluorescence by the mercury lines at 365 mμ).

In addition, a shift of 1.5-2 mμ of the red absorption maximum of chlorophyll toward the longer wavelength region was observed upon the bleaching of the green solution as a result of the decomposition of that form of the pigment which has its maximum at 670 mμ.

The phenomenon is especially apparent in experiments with green solutions of light-green beet leaves and with ordinary leaves in alkaline buffer; under these conditions and upon illumination with red light for 5 minutes, the amount of the bleached form reached 30% of the total amount of chlorophyll. The amount of the labile form differs in various plants: up to 30% of the chlorophyll was bleached in green solutions of beet and poplar leaves during the experiment, while in "solutions" of bean leaves only approximately 3% was bleached.

It should be noted here that the comparative investigation of the kinetics of bleaching of chlorophyll a + b in alcohol-glycerin and plain alcohol solutions has shown the usual type of kinetic curves which are characteristic for true solutions.

* On all figures, the beginning of the curve represents the beginning of the experiment.

The Influence of Preliminary Heating of the Leaves and of the Green Solutions on the Absorption Spectra, the Bleaching of Chlorophyll, and the Photochemical Activity

It has been known for a long time that heating the leaves to 100° results in a shift of the red absorption maximum of chlorophyll toward the shorter wavelength region. It is also well known that heating promptly destroys the ability of a suspension of chloroplasts, as well as of the green solutions, to react in the Hill reaction.

However, in the presence of water-glycerin media an increase in the thermal stability of the enzyme systems may be expected. For this reason, we determined the photochemical activity in green solutions that were heated before the preparation of the green solution.

In these experiments we used green solutions prepared from beet leaves, grown in the winter time close to the window, in a buffer of pH 7. The chlorophyll in these "solutions" usually had its absorption maximum at 676 mμ and was quite stable to the action of light. Upon a 6 minute illumination with red light at 20° only approximately 3% of the total amount of pigment was bleached.

a) Influence of the heating of leaves and of the green solutions on the bleaching of chlorophyll and the position of its absorption maximum.

The rate and extent of chlorophyll bleaching in green solutions are to a large extent dependent on the temperature. Thus, Sidorin [9] observed the decomposition of chlorophyll under the action of sunlight in preliminarily heated soya leaves. In fact, a prolonged heating of the leaves and of the green solutions led to an increase of the fast-bleaching form of chlorophyll and was accompanied by a gradual shift of the absorption maximum of the pigment toward the shorter wavelength region.

Heating to 30° (for 1 hour) of the leaves and of the green solutions caused no noticeable change in the absorption spectra or in the amount of the bleaching form of chlorophyll, in spite of the fact that the rate of chlorophyll bleaching in the green solution is significantly higher at 30°, than at 20°. This may indicate a shift of the balance between the aggregated and monomeric forms during bleaching.

Upon heating the green solution to 40-43° for 30 minutes, the amount of chlorophyll which bleached during 6 minutes increased by a factor of 2-4. Upon heating the solution for 15 minutes to 45° the absorption maximum of chlorophyll shifted to 673 mμ, and the bleaching increased by a factor of 4. A 10 minute heating to 50° caused a shift of the absorption maximum to 671 mμ, and at the same time the bleaching increased by a factor of 6 in comparison with the initial "solution".

The chlorophyll was more stable in the leaves. Heating the leaves to 40-45° for 15 minutes did not change the spectral and photochemical properties of the chlorophyll in green solutions in comparison with the control experiment without the heating of leaves.

Upon heating to 50-52°, the red absorption maximum of chlorophyll was shifted to 674 mμ, and the bleaching increased by a factor of 5-7. After heating the leaves for 15 minutes to 60° the position of the red absorption maximum was at 671 mμ, while after a 5 minute heating to 100° it was at 670 mμ. In the last case, approximately 50% of the total amount of chlorophyll in the green solution bleached during the experiment.

b) Influence of the heating of leaves and green solutions on the Hill reaction.

The water-glycerin green solutions had high photochemical activity. The control experiments in which we investigated the dark reduction of the hydrogen acceptors showed that phenolindophenol was reduced only insignificantly, while dichlorophenolindophenol was reduced rapidly. A preliminary heating of the green solutions (before determination of the photochemical activity) and of the leaves (before preparation of the green solution) significantly increased the amount of substances able to reduce dichlorophenolindophenol in the dark. The green solutions prepared from leaves heated to 100° reduced almost all of the added dichlorophenolindophenol after 3 minutes in the dark as well as in light.

Preliminary heating of the green solutions before the experiment decreased the photochemical activity when measured with dichlorophenolindophenol. Heating the green solution to 40° for 10 minutes decreased the activity by 40% in comparison with the activity of the control experiment, and a 30 minute heating to the same temperature — by 80%.

Heating the leaves before the experiment also decreased the photochemical activity of the green solutions but to a lesser extent than the heating of the prepared "solution".

Upon determination of the photochemical activity with phenolindophenol the heating of the leaves decreased the activity to a much lesser extent. In experiments on green solutions without preliminary heating of the leaves approximately 30% of the added dye was reduced. After preliminary heating of the leaf for 15 minutes to 45° the obtained green solution reduced 17-20% of phenolindophenol, and after heating the leaf to 50° for 5 minutes – 6%.

Therefore, the heating of the leaves and of the green solutions before the experiment to above 40-45° leads to an increase of the amount of the bleaching form of chlorophyll, apparently accompanied by a non-reversible change of its state.

Influence of the Temperature of the Plant Surroundings on State of Chlorophyll

The following experiments were performed on green solutions obtained from leaves of beet plants grown in the field during the months of May and June. It was found that the temperature of the media surrounding the plant and the temperature of the room where the colloidal green solutions were prepared influenced the spectral and photochemical properties of chlorophyll. A decrease in temperature leads to a shift of the red absorption maximum of chlorophyll toward the longer wavelength region of the spectra and to a decrease in the amount of the bleaching form of the pigment; a higher temperature favors a shift of the maximum toward the shorter wavelength and an increase in the amount of chlorophyll which bleaches during the experiment.

The amount of the monomeric, bleaching form of the pigment was increased when the homogenized material was kept at a temperature higher than that under which the plants were kept. This might be the result of a slow disaggregation. Changes of the properties of chlorophyll in green solutions under the action of temperature indicate the possible occurrence of reversible and non-reversible conversions of the various forms of chlorophyll upon change in the conditions of the surroundings.

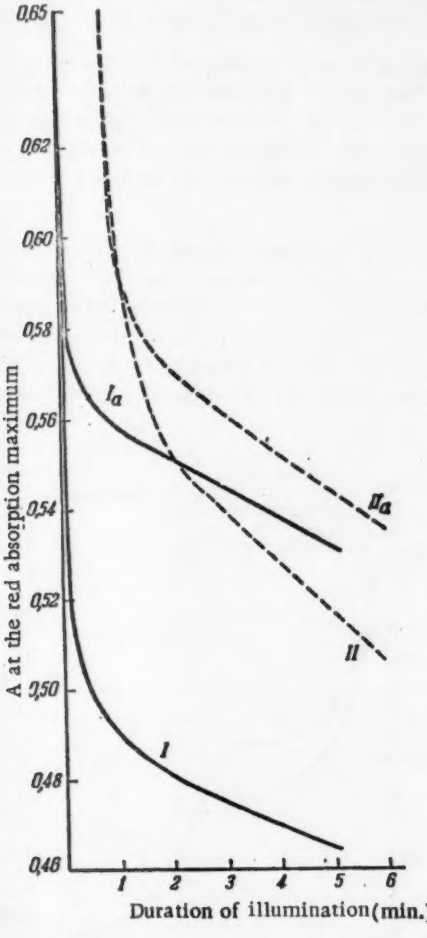

Fig. 2. Influence of the temperature at which the green solution was prepared, on the kinetics of chlorophyll bleaching:
I) Green solution prepared at 20° from leaves grown at 22°; Ia) the same, at 2°; II) green solution prepared at 20° from leaves grown at 15°; IIa) the same, at 2°.

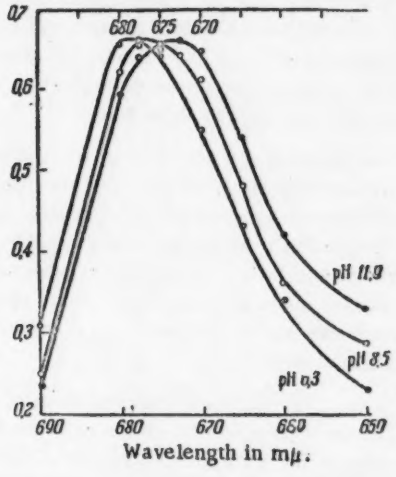

Fig. 3. Influence of the pH on the position of the red absorption maximum of chlorophyll in green solutions.

Fig. 2 shows the kinetics of chlorophyll bleaching in green solutions in a buffer of pH 8.5 prepared at a temperature of 2° (Ia, IIa) and at a temperature of 20° (I, II).

Glycerin, at room temperature, was added to the "solution" prepared at 2°. The bleaching was determined at 20-21°. Expt. 1 was performed on a sunny day (temperature of the air in the morning: 16-17°, in the afternoon: 28°). Expt. 2 was performed on the following day, when a sharp change of the weather had occurred. The day was cold and cloudy; the temperature in the morning was 8-10° and in the afternoon 15°. Upon comparing curves I and II, I and Ia, and II and IIa, the decrease in the amount of the bleaching form of chlorophyll upon a decrease in temperature becomes clearly apparent. The "solutions" prepared on the first and second days and the "solutions" prepared during the same day but at different temperatures differed in the position of their red absorption maximum: I-674 mμ; Ia-675.5 mμ; II-676 mμ; IIa-677 mμ.

Influence of the pH on the Absorption Spectra and the Bleaching of Chlorophyll in Green Solutions

The influence of the pH on the properties of protein solutions is well known; usually, alkaline media favor disaggregation by increasing the solubility of proteins. However, systematic studies of the influence of the pH on the spectral and photochemical properties of chlorophyll in its native state are lacking up to the present time.

Our experiments have shown that the properties of chlorophyll in green solutions of beet leaves vary with the pH of the phosphate buffer used in the preparation of these "solutions". A shift of the pH to the alkaline side induces a shift of the red absorption maximum of chlorophyll toward the shorter wavelength region (Figs. 3 and 4). The red fluorescence of chlorophyll in green solutions is much more definite in alkaline media than at more acid pH. We have already indicated that at a constant pH the absorption maximum may be found at various wavelengths, depending on the conditions surrounding the plant.

Changes of the position of the absorption maximum at various pH are accompanied by changes in the photochemical stability of chlorophyll in green solutions. For instance, at pH 12 six times as much chlorophyll bleaches as bleaches at pH 4.8, and at pH 8.5, 2-4 times as much as at pH 7. In young poplar leaves, 5 times as much chlorophyll is bleached at pH 8.5 as at pH 4.8 (Figs. 4 and 5). It must be noted that in "solutions" prepared from light-green beet leaves the amount of the fast bleaching form of chlorophyll and the position of red maximum vary little with pH within the range of pH 7-8.5 and that at pH 4.8 all of the green substance separates upon centrifugation.

The question of whether any chemical changes occur in the chlorophyll within the investigated pH range then arises. The isoelectric point of the green solution is usually found within the range of pH 4.5-5; during short experiments, it is difficult to assume the occurrence of an extensive pheophytinization which is supported by spectral analysis data.

It is known that a product with a red absorption maximum at approximately 640 mμ forms upon the action of bases on chlorophyll [10]. In order to verify the possibility of the formation of such products in alkaline buffer under the conditions of our experiments, we measured the absorption spectra of green solutions in this region. However, no maximum at 640 mμ was observed.

In the light of the obtained results it became interesting to investigate the influence of the pH on the position of the absorption maximum of colloidal water-glycerin solutions of chlorophyll. In order to obtain the colloidal solution, 0.2-0.4 ml of an alcohol solution of chlorophyll a + b was added to 4 ml of phosphate buffer in such a way that A in the

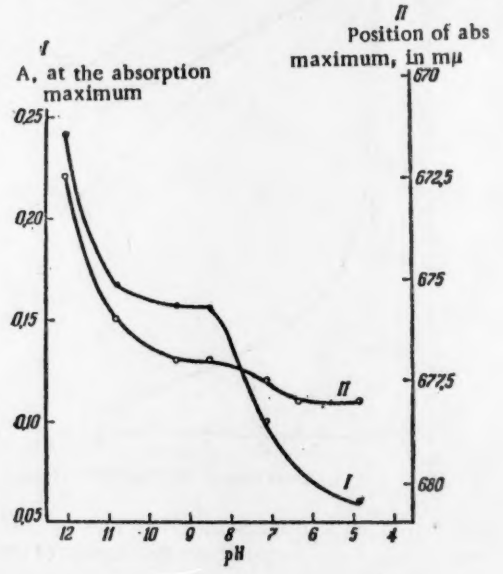

Fig. 4. Influence of the pH on the bleaching and the position of the red absorption maximum of chlorophyll in green solutions.

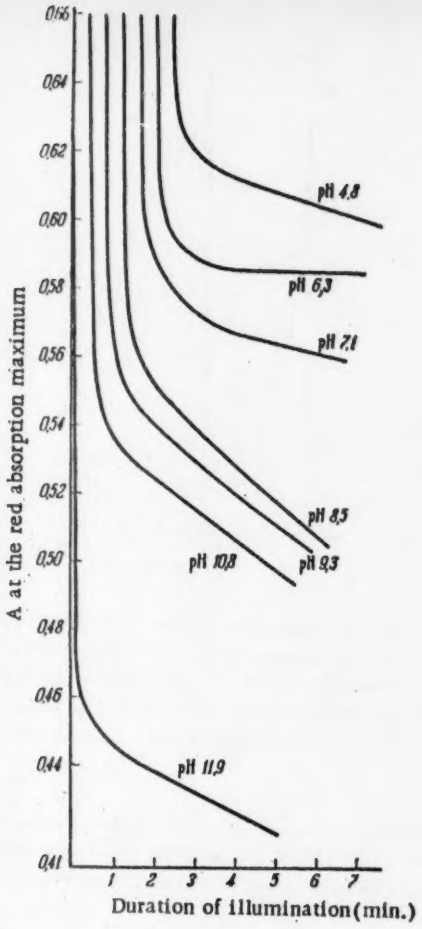

Fig. 5. The bleaching of chlorophyll in green solutions at various pH values.

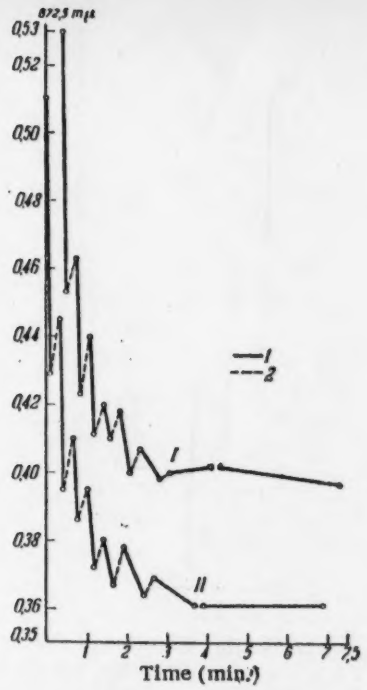

Fig. 6. Reversible oxidation of chlorophyll: 1) light; 2) darkness; I) green solution, pH 8.5; II) same, with the addition of 2.5 mg of neutralized ascorbic acid.

red absorption maximum would be equal to 0.6 upon the addition of 60% glycerin. As in green solutions, a decrease of the pH was accompanied by a shift of the red maximum toward the longer wavelength region of the spectra. The absorption maximum of chlorophyll in the colloidal solution prepared with a buffer of pH 11.9 was found at 671 mμ, and with a buffer of pH 4.8, at 677 mμ. The bleaching of chlorophyll in colloidal solutions also varies with the pH, and decreases with rising acidity of the media.

Reversible Photochemical Conversions of Chlorophyll in Green Solutions

The fact that the green solutions were obtained with large amounts of the active form of chlorophyll made possible their investigation by methods that have been applied in our laboratory for the study of the photochemical properties of true solutions of pigments.

a) Reversible oxidation. The ability of chlorophyll, bacteriochlorophyll and magnesium phthalocyanine to form unstable oxidized compounds which react in the dark with reducers resulting in a partial regeneration of the molecules of the initial pigment, was demonstrated in our laboratory upon illumination in the presence of atmospheric oxygen [11].

To search for similar conversions of chlorophyll in green solutions led to positive results.

The green solutions prepared on a buffer of pH 8.5 were illuminated at 20° in the presence of air and the optical density (A) was measured at the red absorption maximum at the usual times (see above). A fast decrease

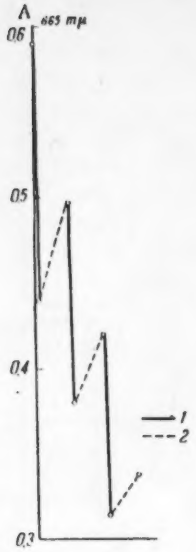

Fig. 7. Reversible oxidation of chlorophyll a + b in a mixture of alcohol and glycerin: 1) 5 seconds illumination; 2) 5 minutes darkness.

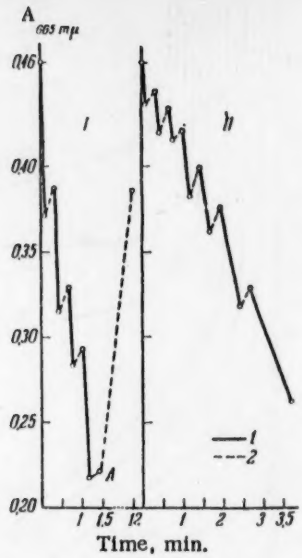

Fig. 8. Reversible oxidation of chlorophyll: 1) Illumination; 2) darkness; I) solution of chlorophyll a + b in a mixture of alcohol and glycerin (ascorbic acid was added at Point A); II) chlorophyll a + b in alcohol.

of A was observed during the first seconds of illumination (Fig. 6). A few seconds (usually 10-15 seconds) after the switching off of the light the absorbance of chlorophyll at the maximum again increased. The same phenomenon occurred upon further periods of light and darkness; the magnitude of the effect progressively decreased upon further illumination. A similar picture of reversible oxidation was observed in the presence of ascorbic acid (Fig. 6).

The ascorbic acid (12.5 mg) was neutralized before the experiment with 2 ml of a saturated Na_2HPO_4 solution; the pH of the obtained solution was 7.5. The solution was mixed with glycerin in a ratio of 4:6. A mixture of 1 ml of water-glycerin solution of ascorbic acid with 3 ml of green solution was prepared for the experiment.

In order to compare the behavior of chlorophyll in water-glycerin green solutions with the behavior of chlorophyll that is not bound to proteins, we performed some experiments on alcohol-glycerin solutions of chlorophyll (a + b). The alcohol solution of chlorophyll (a + b) was mixed with glycerin in a ratio of 3.5 : 6.5 so that the absorbance of chlorophyll at 665 mμ would be equal to 0.6-0.7.

The partial regeneration of chlorophyll in the dark after illumination of the solution in the presence of air is even more apparent in these solutions (Fig. 7). In this case, glycerin is probably the reducer which reacts with the labile, oxidized compounds of chlorophyll. Upon the addition of ascorbic acid to illuminated alcohol-glycerin solutions of chlorophyll a significant regeneration of chlorophyll from the products of its photooxidation occurred. This is in conformity with the observations made in our laboratory during investigation of the photo-oxidation of alcohol solutions of chlorophyll (Fig. 8).

Due to the fast rate of the direct and reversed reactions, we could not measure the full spectrum of the products formed as a result of illumination. However, an increase of the absorption in the region of 510-560 mμ occurs as a result of photooxidation in accordance with previous observations [11]; the presence of ascorbic acid hinders this increase.

In experiments performed on green solutions at −40° the A of chlorophyll at its maximum also showed reversible changes, but the process was much slower than at 20°. It is difficult to determine the course of the

basic photochemical process of electron transfer with the given method of measurement.

b) Reversible photoreduction. A detailed investigation of the reversible photoreduction of chlorophyll in organic solvents has been made in our laboratory (for instance [12, 13]). The reaction may be observed with the aid of a spectrophotometric method in the absence of air, since the photoreduced form of chlorophyll reacts quickly with oxidizers, including oxygen. When this reaction takes place at room temperature there is a sharp decrease of the red absorption maximum, along with the formation of stable photoreduced products. Upon switching off of the light, the reaction goes into the reverse direction relatively slowly. If this reaction takes place at a temperature of −40 to −70°, no stable, reduced products resulting from secondary dark reactions following the action of light accumulate; at low temperature, the formation of an initial photoreduced form of chlorophyll, which is immediately oxidized upon the onset of darkness, may be observed [13].

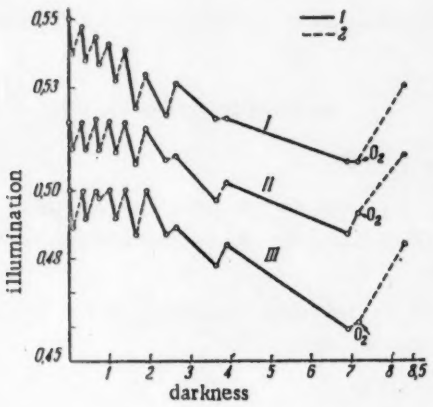

Fig. 9. Reversible reduction of chlorophyll: 1) Illumination; 2) darkness; I) green solution of beet leaves, pH 8.5 (A 672 mμ); II) same, with the addition of 2.5 mg of neutralized ascorbic acid; III) solution of chlorophyll a + b in a mixture of alcohol and glycerin (A 665 mμ).

We attempted to uncover similar conversions of chlorophyll in the green solutions. As reducers, we used the glycerin present in the media and other hydrogen donors or the added ascorbic acid and the neutralized Na_2HPO_4. In these experiments, the vacuum was obtained by lengthy pumping (7-8 minutes) by a vacuum oil pump and by a strong agitation of the sample. Since the media was relatively viscous the removal of oxygen was not as thorough as in experiments with organic solvents. However, in this case, as in all other experiments on the photoreduction reaction, a complete removal of oxygen should be expected as a result of the oxidation of a hydrogen donor, sensitized by chlorophyll [14]. The green solutions were illuminated under the above-described conditions and for the same periods of time. No significant irreversible bleaching of chlorophyll occurred in these experiments; a reversible decrease of the red maximum, which could recur several times upon a short illumination interval, was observed (Fig. 9). A more complete reversibility may be observed in the presence of ascorbic acid. Upon the admission of air into the green solution previously illuminated under vacuum, A usually increased, as a result of the oxidation of the stable, reduced compounds. At −40°, no reversible changes in the green solution in the absence of air could be observed. This agrees with the results obtained in experiments on solutions. The initial photoreduced form of chlorophyll is very active, and is completely oxidized within 10 seconds. The same phenomena are observed in an alcohol-glycerin solution of chlorophyll (a + b) as in the green solution.

It has been demonstrated in our laboratory that the photoreduction reaction is accompanied by a shift in the potential of the inert electrode toward the negative side as a result of the formation of negatively charged electrolytic products of the reduction of chlorophyll [13].

We measured the photopotential of a platinum electrode in the green solution, in the absence of air, according to the method used in our laboratory. In this case also we found that a shift of the electrode potential toward the negative side occurs upon illumination, and that it is quickly reversed in darkness. All these experiments indicate that in absence of air there occurs a reversible photoreduction of chlorophyll by the hydrogen donors present in the media.

SUMMARY

Our experiments demonstrate that the green solutions contain photochemically stable forms of chlorophyll as well as a form which is photochemically active, the amount of which depends on the type of plant, on the conditions surrounding the plant, on the temperature, and on the pH of the buffer solution.

A choice of conditions under which a large amount of the active form is produced (sugar beet, pH 8.5) allowed us to observe reversible photochemical conversions of chlorophyll in its native state.

The active form of chlorophyll is similar in its properties to the "monomeric" solutions of chlorophyll in organic solvents. This form exhibits fluorescence and reversible oxidation-reduction conversions under the action of light. What is the nature of the chlorophyll compound in this state? No reliable data are yet available on this subject, and only the following may be postulated at the present time. In aqueous colloidal solutions, chlorophyll does not exhibit any fluorescence; a bonding with liqids or detergents is necessary for its appearance. This provides for disaggregation, inasmuch as the interaction among adjacent molecules of chlorophyll in the colloidal particle leads to an extinction of the fluorescence and to a shift of the absorption toward the longer wavelength region of the spectra. Thus, the fluorescing, active form of chlorophyll in green solutions must be bonded with lipids of lipoproteins. The determination of the nature of this bond is a problem for further investigations. We have already offered the hypothesis that the variations of fluorescence observed in plant leaves upon changes in the light and darkness regime must be attributed to conversions of this active form of chlorophyll [1, 2, 15].

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DISTRIBUTION OF THE IRON-PORPHYRIN ENZYMES AMONG DIFFERENT PARTS OF BARLEY PLANTS

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Sisakyan and Rubin [1] observed that the relationship between the peroxidase and the polyphenol oxidase activities varies in the leaves of apple trees in accordance with the age of the leaf. Mikhlin and Kolesnikov [2] studying the activity of various oxidases and peroxidases in barley seedlings found that cytochrome oxidase is the prevalent oxidase in this plant at this early stage of development. Later on, at the main stage of biological oxidation, i.e. in the activation of molecular oxygen, the main role is transfered to other oxidases: to the copper-containing oxidase which Mikhlin and Kolesnikov mistake for polyphenol oxidase and which James [3] considers to be ascorbic acid oxidase; while at a later stage of development the main role falls to an oxidase or oxidases which are not sensitive to relatively weak concentrations of cyanides and which are assumed to be flavoprotein oxidases. It was initially demonstrated by Kolesnikov [4] that in the green parts of plants one of the most active of these oxidases is the oxidase of glycolic acid, which couples with pyridine nucleotide coenzymes through the reductase of glyoxylic acid [5]. The observations of Mikhlin and Kolesnikov [2] were supported by Kryukova [6] in experiments on barley, and by Sisakyan and his coworkers [7] in a number of investigations on wheat.

However, while all of these observations may be assumed to represent a relay of enzymes in the whole organism or any of its organs, the results of the present investigation basically demonstrate that simply a differentiation of oxidizing enzymes occurs in accord with differentiation of the organs and tissues in the growing plant organism and the appearance of the functional characteristics of each of its component parts.

Only assumptions and hypotheses exist on the internal relationship between the morphological structure of plant organs or organoids and their function on one band and the function of any oxidizing enzymes on the other. Thus, for instance, Lundegardh [8] relates the activity of the cytochrome system in plant roots to the use of the respiratory energy for the transfer of various anions, against the concentration gradiant, from the surrounding media. However, generally speaking, the assumption that the morphological and functional characteristics determine the main activity of any enzyme in various parts of the plant organism still require experimental evidence.

In the present work we compared the activity of one of the main enzymes of biological oxidation, i.e. cytochrome oxidase, with the activity of other enzymes related to it in chemical composition: catalase and peroxidase. While the chemical nature of the prosthetic group of cytochrome oxidase is not yet known, there is no doubt that this group belongs to the iron-porphyrin compounds which enter into the composition of some other enzymes. A common precursor of cytochrome oxidase, peroxidase and catalase is the porphyrin group or possibly even the whole iron-porphyrin group. For this reason comparison of the activities of the above-mentioned three enzymes may yield some information on the intensity of the whole synthesis of porphyrin compounds in specific plant tissues and on the distribution of these compounds among various hemoprotein enzymes, and also among these enzymes and chlorophyll in the green parts of plants.

EXPERIMENTAL

The material for these investigations was barley of the "Viner" variety, 1954 corp, grown at the Gribovskaya biological station. The barley was grown on filter paper at 20-25°, in diffuse light, in dishes containing distilled water. The experiments were performed on various parts of an 8-day plant. The barley sprout was subdivided into 6 parts (Fig. 1). The parts used in the experiment were: 1 cm of the lower tip of the root, 6 cm of the upper part of the root, the cotyledon, 3.5 cm of the lower part of the leaf, 6 cm of the middle part of the leaf, and 1 cm of the tip of the leaf.

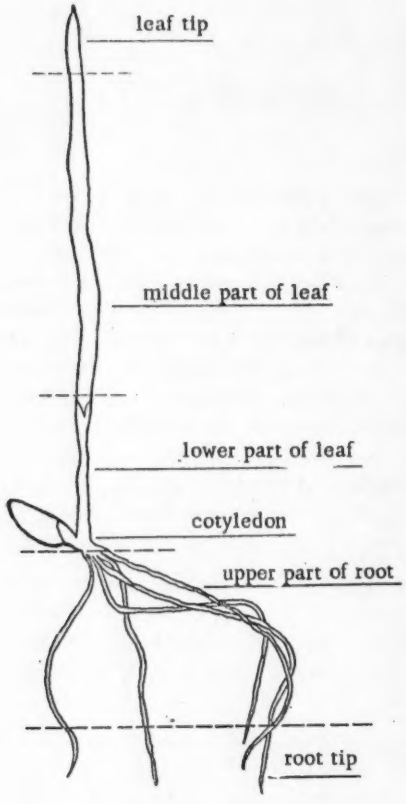

Fig. 1. An 8-day old barley seedling.

Determination of Cytochrome Oxidase

The activity of this enzyme was determined in the Warburg apparatus at 25°. The volume of the reaction mixture was 5.3 ml. 0.7 ml of cytochrome solution ($4 \cdot 10^{-4}$ M) obtained by the method of Keilin and Hartree [9] and 0.3 ml of a solution of hydroquinone (1%) were in the side-bulb arm. The suspension and water were placed in the main part of the vessel. The central part contained 0.3 ml of a 10% KOH solution. The suspension was prepared on phosphate buffer of pH 7.3 (1/15 M). The amount of oxygen consumed during 1 hour was measured.

A colorimetric method [10] for the determination of cytochrome oxidase activity in a suspension of mitochondria from rat kidneys was recently described.

The method is based on the well known formation of indophenol blue from α-naphthol and dimethyl-paraphenylene diamine. An attempt to apply this method to the determination of cytochrome oxidase in suspensions of barley seedlings did not produce satisfactory results. The amount of enzyme preparation and the intensity of the obtained color are out of proportion.

The inherent impossibility or difficulty of application of this rapid and simple colorimetric method of determination of cytochrome oxidase in plants is apparently due to the presence in the plant material of substances that are absent from animal tissues.

Determination of Catalase

A manometric method was applied for the determination of catalase activity. It is necessary to note that some evidence has been published [11] on the non-applicability of this method to studies of the kinetics of the decomposition of hydrogen peroxide by catalase. However, under specific conditions, we succeeded in applying this method for the determination of the relative activity of catalase [12]. The advantage of this method consists in the fact that it does not involve permanganate titration of the stained solution or the liberation of the pigments from the preparations, with the unavoidable partial inactivation of catalase that occurs under these conditons.

We first determined the conditions under which the manometric method would be fully satisfactory. These conditions are as follows. The suspension is prepared in phosphate buffer of pH 7.0 (0.01 M). The material is homogenized in a mortar for 3 minutes. It must be noted that the suspension prepared in water exhibits the least catalase activity. This is in complete agreement with earlier data [13] on the function of phosphate buffer in the determination of catalase activity.

The main part of the vessel contained 3 ml of phosphate buffer, pH 7.0 (0.01 M), 1 ml of suspension and 1 ml of water; the side bulb contained 0.3 ml of H_2O_2 solution (0.02 M). KOH was not used since practically no carbon dioxide is evolved during 1-3 minutes.

The concentration of the plant material in the suspension was determined empirically. 3-12 mg of fresh plant leaf tissue was used in each experiment. In roots where the catalase activity is significantly lower, up to 100 mg of fresh weight was taken. At higher concentrations, the proportionality between the amount of enzyme and the amount of liberated oxygen is disturbed. After stabilizing the temperature for 12 minutes at 25°. in a thermostat, the hydrogen peroxide was admitted from the side-bulb of the vessel into the suspension, and the timer was immediately switched on. No liberation of oxygen was observed in control experiments without enzymes during the same period of time (1-3 minutes).

TABLE 1

Elimination of O_2 from Hydrogen Peroxide in a Homogenate of Barley Leaves (μ l)

Age of plant	Weight of mass (mg)	Time, minutes		
		1	2	3
First leaf of an 8 day seedling	12.5	27.58	70.92	112.23
	3.125		16.74	28.07
First leaf of a 9 day seedling	6.25	13.79	39.40	68.95
	3.125	5.91	21.67	34.47

The experiments showed that the proportionality between the amount of enzyme and the liberated oxygen exists under such conditions. This can be seen from Table 1, where the oxygen liberated is represented in μ l for 1, 2 and 3 minutes in the suspension of barley leaves.

Determination of Peroxidase

We initially attempted to apply a manometric method based on the liberation of carbon dioxide (along with the formation of purpurogallin) upon the oxidation of pyrogallol [14] for the determination of the peroxidase activity. This method was verified on peroxidase isolated from horseradish.. The determinations were performed under optimal conditions according to Wilstätter [15] . The side-bulb contained 0.5 ml of a pyrogallol solution (12 mg) and 0.5 ml of an H_2O_2 solution (0.025%); the main part of the vessel contained 3.0 ml of phosphate buffer, pH 6.2, and 1 or 0.5 ml of peroxidase solution containing 0.1 mg of enzyme per 1 ml.

The results are given in Table 2.

TABLE 2

Elimination of Carbon Dioxide Gas Upon the Oxidation of Pyrogallol by Horseradish Peroxidase

Time (minutes)	10	20	30	40	50	60
Amount of solution (ml)	CO_2 in μ liters					
0.5	5.91	17.73	33.49	45.31	61.07	80.71
1.0	15.76	31.52	45.31	56.73	74.86	92.59

Thus, a strict proportionality between the enzyme concentration and the liberated CO_2 is absent in experiments with the isolated but insufficiently purified peroxidase of horseradish, while satisfactory results are obtained with sufficiently purified enzymes [14].

The following modification of the Wilstätter method [16] proved to be quite satisfactory. This modification, in comparison to others, requires only small volumes of reagents.

A 50 ml Erlenmeyer flask contained: 2 ml of a fresh 5% solution of pyrogallol; 2 ml of 0.5 M phosphate buffer, pH 5.9; 1.5 ml of H_2O and 1 ml of a 1% solution of H_2O_2.

After bringing the temperature of the mixture to 20°, 1.5 ml of suspension (500 mg of fresh mass + + 5 ml of H_2O_2) was added, the timer was switched on, and after 5 minutes the formation of purpurogallin was stopped by the addition of 0.5 ml of a 4N H_2SO_4 solution. The purpurogallin was extracted by ether, the ether solution was suction filtered through a filter paper, and the volume was increased to 50 ml. The determination was performed in an electric photocolorimeter with a light-blue filter. The standard solution contained 12 mg of purpurogallin dissolved in 25 ml of ether.

Determination of Chlorophyll

The chlorophyll in the tip, middle and lower segments of the leaf was determined according to Godnev [17].

For the determination of chlorophyll in the tip and middle segments of the leaf 1 g of material was used, while for the determination in the lower part where significantly less chlorophyll is present, 2 g of fresh material was used. The leaf tissue was rapidly homogenized in a mortar with dry $MgCO_3$, followed with alcohol; the homogenate was then filtered through a glass filter (No. 3). The volume of the filtrate was brought up to 50 ml. The color intensity of the solution and the absorption at 660 mμ were measured in a Beckman spectrophotometer.

DISCUSSION OF RESULTS

Cytochrome Oxidase

There is no longer any doubt that cytochrome oxidase, as well as other oxidase systems, is very widely distributed throughout the plant kingdom. Only its specific significance remains incompletely explored. According to some authors [18] cytochrome oxidase is the main, and possibly the only, functional oxidase in the plant kingdom. Others [2, 3] consider the cytochrome oxidative system to be predominant only at the early stages of plant development. The polyphenol oxidase, ascorbic acid oxidase, or the flavoprotein oxidative systems may be the predominent systems present in the various organs and tissues of the mature plant. Therefore, the problem of a "general" [18] oxidase of plant seems to be irrelevant. This can be seen from the data obtained by us on cytochrome oxidase, the activity of which varies greatly in various plant organs (roots, cotyledon, leaf) and even in various parts of the same organ.

In the determination of cytochrome oxidase activity we accounted for the additional (exogenous) absorption of oxygen which resulted from the addition of cytochrome C to the homogenate where hydroquinone was present as the oxidizing substance. The results are given in Table 3.

TABLE 3

Cytochrome Oxidase Activity in Various Parts of the Barley Plant (μl O_2/100 mg dry wt./hr.)

Leaf tip	Middle part of the leaf	Lower part of the leaf	Cotyledon	Upper part of the root	Root tip
187.0	113.1	117.2	353.1	52.4	377.5

Cytochrome C from beef heart is often used as the direct substrate of vegetable cytochrome oxidase in studies of the activity of this enzyme in plant material. But never has it been demonstrated that the vegetable cytochrome oxidase is identical to the animal cytochrome oxidase, and that in general the components of this oxidative system are the same in plants as in animal organisms. On the contrary, some data [8] indicate a significant difference between the oxidative cytochrome systems in plants and animals, and even between the non-green and green parts of the plants. Some cytochromes that are absent from yeast and animal organisms were found in chlorophyll-containing parts of plants [18] as well as in photosynthesizing bacteria [19]. Their biological functions remain unknown.

It can be seen from Table 3 that the highest cytochrome oxidase activity of all the investigated parts of barley is found in the root tip. While the activity of this enzyme is also very high in the cotyledon, it is somewhat lower than in the root tip. The activity in the upper segment of the root is only 15.5% of the activity exhibited by the root tip.

The cytochrome oxidase activity also varies in the various parts of the leaf; the activity is almost the same in the middle as in the lower segments of the leaf. The cytochrome oxidase activity in the young and rapidly growing segment is greater than in the remaining parts of the leaf by a factor of approximately 1.5.

It must be noted that James and Boulter [20] did not find any cytochrome oxidase in 7-day barley seedlings, and that Mikhlin and Kolesnikov [2] found that the cytochrome oxidase activity in 6 or 7-day seedlings was very weak. In the above-described experiments, the enzyme activity, while being very weak, persisted at least to the 8th day. Such a discrepancy may be due to the different varieties of barley that were used [21] or to the conditions under which the plants were grown [8].

Catalase

The problem of the possible relationship between the chlorophyll content of the leaf and the amount or activity of the catalase present in it has long attracted the attention of investigators concerned with the possible function of this enzyme in photosynthesis.

It is considered that the highest catalase activity is exhibited in the green parts of the plants. However, the results of our experiments show that a relatively very high catalase activity is exhibited only in homogenates of young, very rapidly growing leaf tissues. As can be seen from Table 4, the catalase activity in the oldest part of the leaf is only about half as high as in the cotyledon, and almost the same as in the lower segment of the root.

TABLE 4
Comparative Activity of Catalase in Various Organs and Tissues of Barley Plants (μl O_2 per 1 mg of dry substance per 3 min.)

Leaf tip	Middle part of the leaf	Lower part of the leaf	Cotyledon	Upper part of the root	Root tip
256.0	134.1	45.6	78.7	12.3	42.9

The difference in the activity of catalase in two segments of the same leaf may be related to differences in the intensity of oxidative exchanges in these tissues. A more active metabolism is reflected by a more intensive oxygen absorption in young and developing tissues as compared to older ones.

A more or less important function is carried out in the oxidative metabolism by flavin oxidases, such as the well known xanthine and aldehyde oxidases, the oxidases of amines, amino and glycolic acids, as well as the newly discovered DPN oxidase [22]. All of these enzyme systems produce hydrogen peroxide as a by-product. The formation of hydrogen peroxide in living cells, during the process of respiration and the role of catalase, were very convincingly demonstrated by Chance [23]. The relationship between the content of flavoproteins in barley roots and the activity of peroxidase was also observed [8]. It is difficult to understand, in the light of the accumulated facts, the still expressed doubts on the biological function of the catalase reaction and the attempts to limit this function to the oxidation of ethanol which is not found on the main metabolic routes in plant and especially animal organisms, and for the oxidation of which a very active alcohol dehydrase is present in living matter. The peroxide oxidation of other substances in addition to ethanol, i.e. nitrite, with the aid of catalase, is not likely to occur under the existing relatively small concentrations of this enzyme in plants. The doubt expressed earlier by Mikhlin [24] as to the predominant and even exclusive peroxidase function attributed to catalase has at last been fully confirmed by one of the recent investigations of Keilin and Hartree [25], who have found under optimal conditons of peroxidase oxidation of ethanol, that this function is weaker than the catalase activity proper by a factor of 12,000, i.e. than the decomposition of hydrogen peroxide into water and molecular oxygen. The main function of catalase in animal organisms is undoubtedly a defensive function against the detrimental action of hydrogen peroxide which is formed during the respiratory process.

Peroxidase

The decomposition of hydrogen peroxide in plant organisms is catalyzed preferentially by peroxidases which are more or less specific to various natural substrates [26]. A prominent place among these plant enzymes is held by that peroxidase which in combination with polyphenols can oxidize, through the corresponding quinone reductase and pyridine nucleotide, various products of the degradation of hydrocarbons, proteins, and fats. In some plant tissues, for instance in barley roots, the content of this peroxidase amounts to 50% of the content of all hemo compounds [8]. However, other peroxidases may under some conditions act as the main enzymes of biological oxidation. An example may be the cytochrome C peroxidase, the action of which, in the experiments of Mikhlin and Bronovitskaya [27], was related to the function of various enzyme systems which form hydrogen peroxide or organic peroxides.

An intensive oxidation of pyridine nucleotides by the peroxidase system in living yeast cells was demonstrated by Chance [28]. In these experiments, as in those of Mikhlin and Bronovitskaya, the oxygen donor was the organic peroxide: methyl hydroperoxide. Cytochrome C may be oxidized with a significantly lower intensity by other peroxidases [29] as well as by a specific peroxidase.

TABLE 5

Peroxidase Activity in Various Tissues of Barley Expressed by the Purpurogallin Number

Leaf tip	Middle part of the leaf	Lower part of the leaf	Cotyledon	Upper part of the root	Root tip
0.63	0.67	0.8	0.44	0.5	0.9

TABLE 6

Chlorophyll Content in Various Parts of the Barley Leaf (mg per 1 g of dry weight)

Leaf tip	Middle part of the leaf	Lower part of the leaf
0.75	0.53	0.12

Since the prosthetic group of peroxidase as well as cytochrome oxidase and catalase is an iron-porphyrin compound, activity of peroxidase in various plant tissues was studied along with other mentioned enzymes. This study appeared of even greater interest since Lundegardh [8] observed an inverse relationship between the content of peroxidase and the concentration of some of the components of the cytochrome system in roots; he even admitted the possibility of an interconversion of the various iron-porphyrin compounds, depending on the age of the plant and the surrounding conditions.

As can be seen from Table 5 the highest activity of peroxidase in relation to pyrogallol is observed in the lower part of the root, where the most intensive metabolism takes place; the peroxidase activity in another metabolically active tissue is very close to it – that in the tip of the leaf. However, in the cotyledon, where an intense activity of catalase and especially of cytochrome oxidase is observed, the activity of peroxidase is lower than in other plant tissues.

Chlorophyll

The coincidence of the routes of the formation of iron-porphyrin and magnesium-prophyrin compounds in plants may be considered as demonstrated [30]. The common precursor of hemoprotein enzymes as well as of chlorophyll is probably protoporphyrin. For this reason, we determined in various segments of barley leaf the relative content of chlorophyll (a magnesium-porphyrin compound) along with the relative activity of iron-porphyrin enzymes (cytochrome oxidase, catalase and peroxidase).

The results are given in Table 6.

The obtained data show that the chlorophyll content increases from the base to the tip of the leaf. The activity of catalase and cytochrome oxidase changes in the same direction. In regard to catalase, it has been shown [31] that the conditions inhibiting or decreasing the intensity of photosynthesis in the green leaf (low temperature, darkness, magnesium insufficiency) lead to a decrease in the activity of catalase at this site.

The Overall Activity of Metal-Containing Enzymes in Various Parts of the Plant

As we know, cyanides supress the activity of all metal-containing oxidases but to various extents. For this reason, by comparing the degree of inhibition of oxygen absorption in a homogenate prepared from various

plant tissues which results from the presence of a known concentration of KCN, it is possible to obtain some information on the specific function of metal-containing oxidases in the endogenous respiration of various tissues. A uniform method of obtaining such preparations and a careful mincing of the material significantly decrease, if not completely remove, the differences in permeability, which may become decisive factors when whole organs or non-homogenized tissues are used [32].

It was found that an inhibition of the endogenous respiration by a relatively high concentration of cyanide (0.01 M) while occurring to various degrees, was obtained in all the tissues studied. The least effect of cyanide was observed in three preparations, which exhibited the greatest endogenous respiration upon the addition of cytochrome C namely in homogenates of cotyledons and leaf tips (see Table 7).

This discrepancy between the activity of cytochrome oxidase and the degree of inhibition by cyanide demonstrates the fact that upon the activation of oxygen during the respiration of various tissues a function of variable importance is performed by oxidases that are less sensitive to cyanide than cytochrome oxidases. The experiments were performed at pH 7.2. As we know [32], the very weak sensitivity to cyanide of the respiration of the leaf at this pH was explained by an insufficient permeability of the tissue. But the oxygen absorption in a leaf homogenate, where the problem of permeability is irrelevent, was also not sensitive to 0.001 M cyanide.

TABLE 7

KCN Inhibition of O_2 Absorption in Homogenates of Various Tissues from Barley Plants

Part of the plant	μ l of O_2 per hour per 100 mg dry weight		Inhibition (%)
	Endogenous respiration	with KCN	
Leaf tip	75.2	33.7	66
Middle part of the leaf	66.5	18.2	73
Lower part of the leaf	130.2	34.3	74
Cotyledon	153.0	53.4	65
Upper part of the root	42.0	4.2	90
Root tip	81.6	8.1	90

It could be assumed that the absence of the inhibition of respiration upon the addition of cyanide is due to the use of this cyanide in some other reactions besides that of the bonding of cytochrome oxidase. In the first place, this reaction could occur with carbonyl compounds. However, as shown in experiments with semicarbazide it does not increase the influence of cyanide on the respiration of leaf homogenate.

	Control, without additions	+ 0.01 M KCN	0.01 M KCN + 0.01 M semicarbazide
O_2 Absorption (μ liters per g) for 1 hour	78.5	22.5	17.6

Therefore the influence of the carbonyl compounds which could have been present in the homogenate on the inhibition of cyanide is excluded. It remains to admit that a significant amount of the endogenous respiration in leaves is carried out by oxidases which have little sensitivity to cyanides (flavoproteins).

In regard to the respiration in barley root homogenates, it is almost totally (90%) inhibited by 0.01 M KCN in complete accordance with the data of Lundegardh [8] obtained in experiments on whole roots.

DISCUSSION OF RESULTS

The problem of the existence of a predominant or "general" [18] oxidative system in the plant kingdom is more and more often raised in the biochemical literature.

Instead of the polyphenol oxidase system, which was considered for a long time to be the most widely distributed system among plants and the significance of which as a most important oxidative system has found new support in the lately discovered quinone reductase [33], which serves as a link between the polyphenol oxidase system and the pyridine coenzymes, the cytochrome system is held to be the main, or even the only functional oxidative system in plants. At the same time, the very raising of the problem of the

predominance or of the exclusive function of one or the other oxidase system throughout the whole plant kingdom, or even in various families and varities of plants, appears unfounded.

In order to solve the problem of the biological significance of some oxidases, a number of investigators have studied the content of a definite enzyme in various plant varieties. It is clear that these data, often obtained by faulty methods, are insufficient for the drawing of final conclusions. These data are mainly concerned with the determination of the total activity of the enzyme in the whole plant. The fact that various oxidase systems may be synthesized and be functional during the differentiation of the form and functions of individual organs and tissues as well as of the intracellular structures and of the metabolic characteristics related to this differentiation in various parts of the organism, was overlooked. The determination of the total activity of an enzyme in the whole organism cannot give a true representation of the biological significance of that enzyme and of its role in the various functions of the organism. This role may be studied and appreciated only in relation to the metabolic characteristics of individual organs and tissues. It is known, for instance, that the iron-porphyrin enzymes depend on the exchange of iron in the leaves [34], that a relationship exists between the polyphenol oxidase in plants and the content of copper in the plant roots [35], etc.

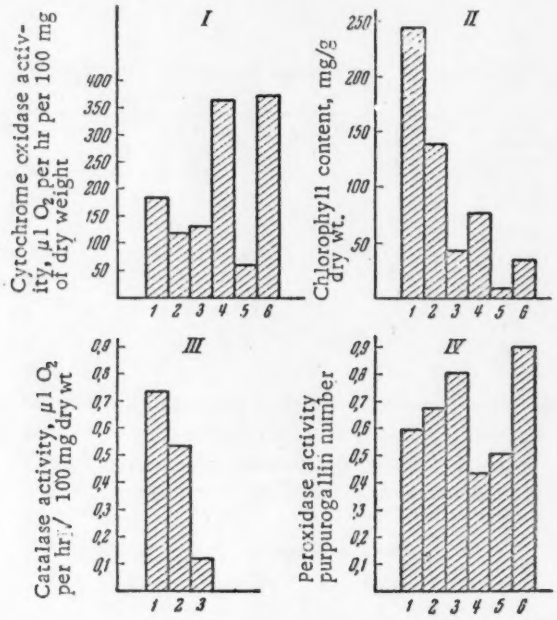

Fig. 2. The activity of iron-porphyrin enzymes and the chlorophyll content in various parts of an 8-day barley seedling.
1) Leaf tip; 2) middle part of the leaf; 3) lower part of the leaf; 4) cotyledon; 5) upper part of the root; 6) root tip.

Within the family of iron-porphyrin enzymes some relationship must exist between the various members of this group, since they all form from the common reserve of porphyrins and probably even from a common precursor protohematin. In addition, the main bulk of porphyrins is used in the synthesis of chlorophyll in the green parts of plants.

Considering that all these possible relationships are undoubtedly present, but that they are little studied, we investigated, as a first approach to the study of the relationship between the various hemoprotein enzymes in an 8-day barley seedling, the comparative activities of cytochrome oxidase, catalase and peroxidase in various organs of the plant (tissues from the lower root tip and the upper segment of the leaf, the main part of the root and also the cotyledon). In addition, the activity of these enzymes in the leaf was correlated with the content of chlorophyll. It could have been assumed that the activity of each of the named components in the various parts of the plant is limited by the competition for porphyrins or protohematin. However, this activity may also depend on the general intensity of the biosynthesis of porphyrin or iron-prophyrin compounds.

It is shown on Fig. 2 that a direct correlation between the activities of cytochrome oxidase and catalase, as well as the content of chlorphyll in the various parts of the leaf, is present in all of the organs and in individual parts of the same organ (leaf and root) of the barley seedlings that we investigated. No correlation could be determined between the activity of peroxidase and that of other porphyrin enzymes or the content of chlorophyll.

The activity of cytochrome oxidase, determined by the additional (exogenous) absorption of oxygen in the presence of cytochrome C and hydroquinone, varies greatly in various parts of the plant. The tissues may be classified according to the activity of this enzyme: root tip > cotyledon > leaf tip > middle part of the leaf > lower part of the leaf > upper part of the root.

However, according to their sensitivity to 0.01 M cyanide the members of this series are classified in a different order: root > lower part of the leaf > middle part of the leaf > tip of the leaf > cotyledon.

Such a poor correlation of the activity of cytochrome oxidase and of the sensitivity of respiration to KCN in various tissues testifies to the fact that other important oxidases which differ from cytochrome oxidase, are functional in the activation of O_2. The polyphenol and ascorbic acid oxidases are the first candidates. However, some flavoprotein oxidases [36] may also be inhibited to a certain extent by a relatively high concentration of cyanide. Lower concentrations, of the order of 10^{-3} M, which almost totally inhibit the action of isolated cytochrome oxidase, exert little influence on the oxygen absorption by homogenates of various tissues of barley seedlings. The very small sensitivity of some plant tissues to weak solutions of cyanide was observed in other experiments [32]. However, this could have been the result of an insufficient permeability of these tissues to cyanide. In our experiments, we used a thoroughly homogenized mass where the contact between the poison and the cytochrome oxidase was facilitated. Therefore, the so called "basic" respiration observed by Lundegardh [8] occuring apart from the cytochrome system takes place not only in barley roots, but also in leaves or at least in specific segments of the leaf and root. The absence in our plant preparations of substances, primarily carbonyl compounds, which could have preferentially attracted the cyanides before cytochrome oxidase can be assertained by the fact that semicarbazide did not influence the oxygen absorption of these preparations. The influence of the carbonyl compounds, if they were present in the preparations, should have been reduced by semicarbazide.

Various oxidases are active in the respiration of the whole plant. The extent to which each of the oxidases is functional in the whole process of respiration which occurs in various segments of the plant depends on the physiological state and the metabolic character of the given structure, as well as on the surrounding conditions. At the time when biochemistry is shifting its interest from cellular physiology to the physiology of intra-cellular structures (plastids mitochondria, microsomes, etc.) the discussion of a predominant and even of a single oxidase appears to be outdated.

SUMMARY

The activity of cytochrome oxidase in various parts of an 8-day barley seedling coincides with the activity of catalase. The highest activity of the above named enzymes is observed in the metabolically most active segments; in the cotyledon, in the upper segment of the leaf, and in the lower tip of the root.

The chlorophyll content in various parts of the leaf coincides with the activity of cytochrome oxidase and catalase in these parts. However, there is no correlation between the activities of the above named enzymes nor between the content of chlorophyll and the activity of peroxidase.

The respiration of homogenates from tissues of various barley organs exhibits an uneven sensitivity to cyanide. This discrepancy cannot be explained by the uneven permeability to cyanide, since the degree of homogenization has no influence. In some tissues, the predominant role in the process of respiration belongs to metal-containing oxidases (cytochrome oxidase, and ascorbic acid oxidase) and in others to flavoproteins, which are little sensitive to cyanide.

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THE BIOLOGICAL ACTION OF VITAMIN P

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We studied the biological action of vitamin P on experimental animals, white rats. The source of vitamin P was a catechin preparation obtained from tea leaves at the A. N. Bakh Institute of Biochemistry, Academy of Sciences, USSR.

According to the data of Kursanova, Bukina, Zaprometova and others [1, 2], the catechin preparation exhibits significant vitamin P activity, and also inhibits the hyperfunction of the thyroid gland. The influence of catechins on the activity of the thyroid gland was demonstrated in the investigations of Gabe and Parrot [3].

We investigated the action of various doses of catechin preparation on the growth and appetite of experimental animals, the ability of the preparation to act on the capillary permeability, and its influence on the thyroid gland.

The capillary reaction was measured by the length of time required for the appearance of hemorrhagic spots (petechiae) under the action of a negative pressure on the shaved surface of the skin induced by a vacuum suction. As a preliminary, we studied some problems of technique, such as: the influence on the capillary reaction of the location of the vacuum suction along various sectors of the skin, the strength of the negative pressure, and the dynamics of the capillary reaction.

We came to the conclusion that the petechiae should be observed at the posterior end of the dorsal side since in this case, upon the shaving of the fur and the application of the vacuum suction the animals are in a more natural position and the obtained data is more uniform than when the suction is applied on the ventral side. In addition, considerations of a general physiological character prevent application of vacuum suction on a soft surface of the body.

Observations of the dynamics of the capillary reaction were performed on rats which were kept on the usual food ration. They received bread, meat, groats, milk, oats, sunflower seeds and vegetables (which are the source of flavones). Two groups of rats were selected for the observations.

In one group of rats (10 animals) the dynamics of the capillary reaction was measured on one sector of the skin after a short interval of time: after 6-7 days for 1 1/2 months; in the other group of rats (15 animals) the dynamics of the capillary reaction was measured after a longer interval of time: 1-2-4 months. Our observations have shown (Table 1 and 2) that in both groups of rats, with periodic measurements of the capillary reaction after a short interval of time (6-7 days) and after a longer interval of time (1-2-4 months), the time required for the appearance of petechiae increased with large individual variations. After observations lasting for 3-4 weeks on both groups of rats it was determined that the time required for the appearance of petechiae increased in all animals in comparison with the initial time, except in one rat. In some animals this increase was quite significant: a few seconds (initial determination) to three minutes and more. 2-4 months later the time required for the appearance of petechiae was also higher than the initial time. However, individual variations were not as great, and the maximal increase did not exceed 2-2 1/2 minutes.

It follows from the obtained data that the duration of the experiment should not be less than 3-4 weeks, a period after which a uniform capillary reaction may be observed in a great majority of rats (in this case

TABLE 1

The Dynamics of the Capillary Reaction in Rats (observations over a period of 1 1/2 months)

Rat No.	Date of experiments (1954)							
	4/V	10/V	16/V	24/V	1/VI	7/VI	14/VI	19/VI
	Time at which the petechiae appeared (sec)							
1	40	8	35	20	50	75	45	72
2	180	40	87	78	45	50	28	19
3	10	6	90	50	22	23	35	18
4	23	10	35	40	100	30	131	85
5	17	45	35	90	55	157	49	34
6	18	22	60	35	40	60	44	84
7	18	13	40	23	20	30	51	38
8	11	180	100	57	180	40	67	42
9	14	29	180	180	83	62	180	44
10	40	58	21	25	12	52	101	61

higher than the initial). The presence of large individual variations in the capillary reaction requires experiments on a large number of animals. Prolonged observation of the character of the dynamics of the capillary reaction demonstrated that the capillary permeability decreased to a certain extent with time.

In all experiments, the capillary reaction was measured under a negative pressure approximately 200 mm lower than the atmospheric pressure; a higher atmospheric pressure in the vacuum sucker (100-150 ml lower than atmospheric pressure) did not influence the character of the capillary reaction.

TABLE 2

The Dynamics of the Capillary Reaction in Rats (observations over a period of 4 months)

Rat No	Date of experiment (1954)			
	5/7	6/7	7/7	9/7
	Time for appearance of petechiae (sec)			
1	7	180	15	45
2	6	180	40	46
3	7	180	160	86
4	10	105	42	126
5	10	135	35	96
6	9	75	35	—
7	7	9	80	84
8	13	40	30	138
9	10	25	105	36
10	14	115	18	—
11	8	30	32	51
12	11	18	90	64
13	8	25	32	58
14	6	14	135	120
15	7	37	12	23

In order to study the biological action of the catechin preparation on the growth, appetite and the capillary reaction, we selected from various families male rats weighing 70-90 g, of approximately the same age, and which were previously kept on a total food ration. Three doses of the preparation were tried: 0.1, 2 and 10 mg, each dose being given to 25 rats. In addition, a control group received no vitamin P. A total of 100 rats were used in the experiment.

The capillary reaction in the skin of the experimental animals was measured at the beginning and at the end of the experiment, by the time required for the appearance of petechiae on not less than three animals.

It must be noted that when the initial capillary reaction had been determined, those rats which the time required for the appearance of petechiae was insignificant were placed preferentially in that group on which we subsequently tried various doses of preparation, while those animals which required a longer period of time for the appearance of petechiae were placed in the control group.

In this way we expected to obtain a more conclusive demonstration of the effect of vitamin P and of the vitamin P-deficient diet on the capillary permeability.

Those rats which exhibited sharp individual variations of the initial capillary reaction were discarded.

During the experiment, which lasted one month, all the rats received a synthetic diet in the form of tablets without any limitations, and in addition, the following vitamins: A 10 units, and D 5 units. Vitamins A and D were administered orally, with a pipette, in an oil solution and the catechin preparation in a water solution.

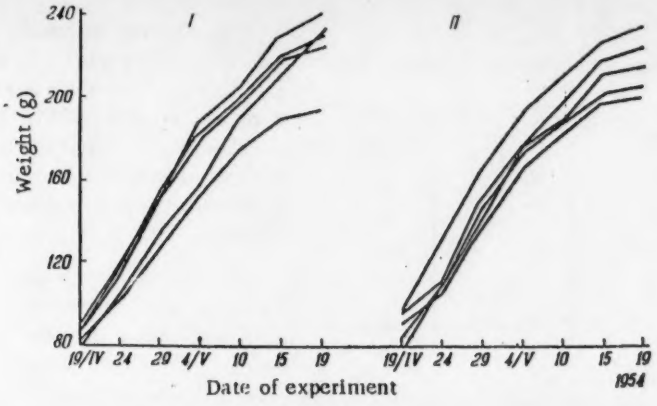

Fig. 1. Weight curves of rats receiving: I) 2 mg of catechin preparation; II) diet without vitamin P.

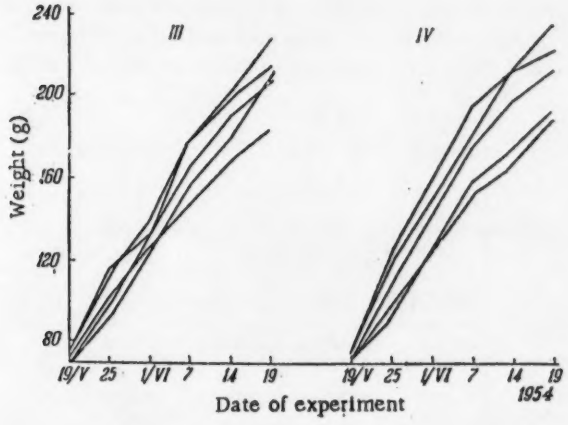

Fig. 2. Weight curves of rats receiving the catechin preparation: iII) 0.1 mg; IV) 10 mg.

Composition of the synthetic diet: Corn starch 65% , casein 18%, bakers yeast 8%, lard 5%, salt mixture (Osborne-Mendel) 4%. The vitamin preparations which were administered to rats were analyzed for their content of vitamins A and D, while the bakers yeast was analyzed for vitamins B_1 and B_2. For the determination of the effect of the preparation on the increase in weight and appetite of the experimental animals, the rats were weighed once in every 5-7 days, and day-to-day account was kept of the remaining food. The weight increase observed during the experimental period is given in Table 3. It shows the average weight of 25 animals by groups at the beginning and the end of the experiment.

As an example we give the weight curves of 5 rats from each group (Figs. 1 and 2). The obtained data on the weight increase and the final weight of rats which received a synthetic diet with vitamin P (various doses) and a vitamin P deficient diet were the same (starting with an identical initial weight).

Table 4 presents the data on the amount of food eaten by the control rats and the experimental rats which received various doses of vitamin P (catechin) during 2 weeks of the experiment. It is seen from Table 4 that the rats kept on a diet with or without vitamin P ate approximately the same amount of food.

From the obtained data it follows that the catechin preparation exerted no specific influence on the weight or appetite of the experimental animals.

TABLE 3

Weight of Rats at the Beginning and End of the Experiment (g)

Date of experiments (1954)	5/18-6/21		6/17-5/18	
Doses of catechin preparation (mg)	0.1	10	2	Diet without vitamin P
Weight of rats at beginning of experiment	78.7	80.9	83.8	82.2
Weight of rats at end of experiment	202.4	202.4	219.9	221.1

The capillary reaction to the negative pressure in rats which received various doses of vitamin P and in control rats was as follows: 92% of the rats which received a 10 mg dose of catechin preparation exhibited an increase in the time of petechiae appearance, while in 8% (2 rats) the capillary reaction did not change (Fig. 3).

Of those rats which received doses of 2 and 0.1 mg of catechin preparation, 56% of the animals in each group exhibited an increase in the time required for the appearance of petechiae; the increase was greater in those rats which received a 2 mg dose than in those which received a 0.1 mg dose; in all remaining animals of both groups it decreased or remained without changing (Figs. 4 and 5). In the control group, the time of petechiae appearance significantly decreased in all rats except one, the capillary reaction of which did not change (Fig. 6). Therefore, the influence of the catechin preparation on the capillary reaction in experimental animals depended to some extent on the dose of vitamin P which they received; the 0.1 mg dose exerted the least influence (only in half of the animals did the time of petechiae appearance increase), the 10 mg exerted a more significant influence, since the time required for petechiae appearance increased in almost all rats; the 2 mg dose exerted an intermediate influence. The time required for petechiae appearance significantly decreased in the control animals deprived of vitamin P.

TABLE 4

Amount of Food (g) Eaten by the Rats During a Two-Week Period of the Experiment

Rat No.	Date of experiments (1954)			
	21/IV—4/V		19/V—1/VI	
	Diet without vitamin P	2 mg of the preparation	0.1 mg of the preparation	10 mg of the preparation
1	288	286	304	314
2	278	272	293	304
3	292	299	288	291
4	286	283	326	268
5	279	313	301	287
6	289	288	282	302
7	301	277	297	304
8	295	302	305	275
9	302	254	296	300
10	266	273	296	289
Average per rat	287.6	284.7	298.8	293.4

The conclusion, that while vitamin P did not influence the growth of the experimental animals, it did exert a definite action on the capillary permeability, may be drawn from our experiments.

The action of the catechin preparation (various doses) on the condition of the thyroid gland of the rats was observed in 3 series of experiments. In the first series of experiments the rats (weighing initially 80 g) received 10 and 0.1 mg of catechin preparation (each dose was given to 15 rats). In the second series (initial weight of rats, 40 g) the experimental animals received 10 mg of catechin preparation, while the control rats received a diet without vitamin P (10 animals in each group). In the third series one group of rats received an even larger dose of catechin preparation than in the first two series (15 mg); the other group received a 0.03 mg dose, while the control group received a diet without vitamin P. Moreover an additional group of rats of the same age and weight as the experimental group was kept on a whole diet (6 rats in each group).

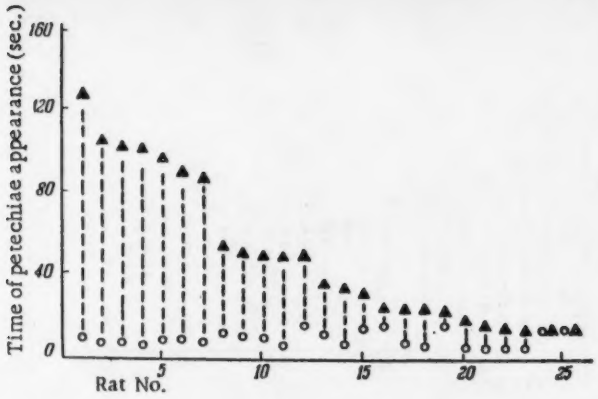

Fig. 3. The capillary reaction in rats receiving 10 mg of catechin preparation: O) Time required for petechiae appearance at beginning of experiment (seconds); ▲) at end of experiment.

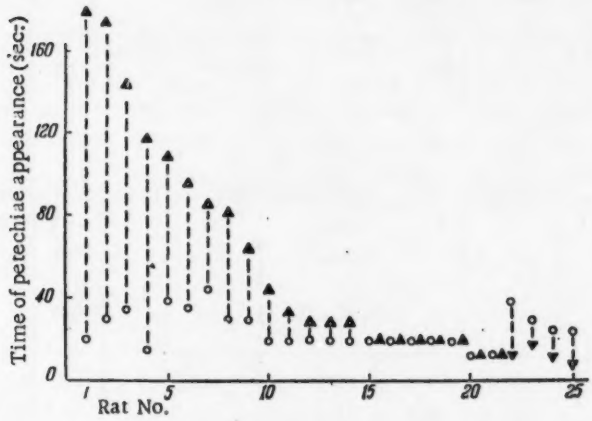

Fig. 4. The capillary reaction in rats which received 2 mg of catechin preparation: O) time of petechiae appearance of beginning of experiment (seconds); ▲) at end of experiment.

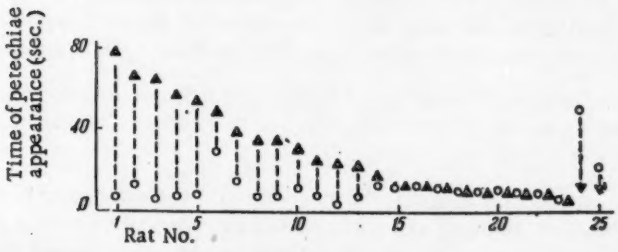

Fig. 5. The capillary reaction in rats receiving 0.1 mg of catechin preparation: O) time of petechiae appearance at beginning of experiment (seconds); ▲) at end of experiment.

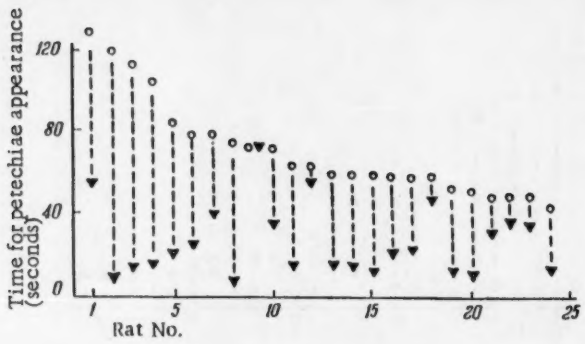

Fig. 6. The capillary reaction in rats receiving a diet without vitamin P (controls): O) time required for petechiae appearance at beginning of experiment (seconds); ▲) at end of experiment.

TABLE 5

Weight of Thyroid Glands of Rats (Absolute, and per 100 g rat weight)

Dates of experiment	Series I		Series II		Series III				Series IV	
	5/21-6/21 1954		10/5-11/4 1954		1/20-2/24 1955				7/27-8/31 1954	
Regimen	Rats received diet and catechin preparation		Rats received diet and catechin preparation		Rats received diet and catechin preparation				Rats received diet without vitamin P	
Weight of thyroid glands	0.1 mg	10 mg	10 mg	Diet without vitamin P	0.03mg	15 mg	Diet without vitamin P	Food ration	With the initial weight (g)	
									40	80
Absolute	21.4	20.9	18.8	18.5	20	19	19	40	18.4	19.4
Per 100 g rat weight	10.4	10.2	12.1	11.6	11.3	10.7	11.5	27.6	10.5	9.5

In order to observe the effect of a diet lacking in vitamin P on the condition of the thyroid gland of rats of various ages, we performed additional experiments (Series IV) in which the rats received only a vitamin P deficient diet (15 rats weighing 40 g and 15 rats weighing 80 g). We carried out the experiment on rats of various ages, since the investigation of the biological action of the preparation of catechin was mainly on rats initially weighing 80 g, while the thyroid gland is most sensitive in younger animals (initial weight 40 g). All series of this experiment lasted for 30 days, the animals were then killed, the thyroid glands removed and weighed.

The histological analysis of the glands of the rats of Series I and II was performed at the Endocrinological Institute of the Ministry of Public Health by E. I. Tarakanov and A. E. Rabkina. The remaining rats were analyzed by I. S. Dergachev.

It follows from the obtained data (Table 5) that the absolute and the relative weights of the thyroid glands of rats which received various doses of vitamin P and of the control animals which received vitamin P deficient diet was the same. At the same time, the absolute and the relative weights of the thyroid glands of the rats from the group which received a whole diet was more than twice the weight of the glands in the experimental animals.

The histological analysis of the rats of Series I and II showed that the rats which received vitamin P, as well as the control rats, had a quiet hyperfunctioning gland, apparently resulting from the special conditions under which the animals were kept. Under ordinary conditions, the thyroid gland appears to be in a somewhat excited state. A normal condition of the thyroid gland was observed in the control animals of the third series which did not receive any vitamin P. A hyperfunction of the thyroid gland was determined in the animals receiving a

whole diet. A tendency toward a hyperfunctioning of the thyroid gland was observed in rats which received 0.03 and 15 mg of vitamin P. This tendency was greater in those rats which received a 15 mg dose of vitamin P. The morphological information on the condition of the thyroid glands is absolutely identical, does not indicate any differences and points to a hyperfunctioning of the thyroid gland in both groups of animals from the fourth series.

Thus, our data on the investigation of the thyroid gland shows that our synthetic diet establishes a quiet hyperfunctioning of the thyroid gland in the experimental animals. Under such conditions the vitamin P in the small doses of catechin preparations (0.03-0.1 mg) as well as in the relatively large doses (10 and 15 mg) had no specific effect on the thyroid gland, regardless of the weight of the rats; the weight of the thyroid gland as well as the histological observations, were the same in rats which received a synthetic diet with or without vitamin P. Only in rats receiving a 15 mg dose of catechin preparation was a significantly greater tendency to thyroid hyperfunctioning observed. In the group of rats which received a whole diet the weight of the thyroid gland was significantly higher, as compared to that in the experimental animals, and the morphological observations showed an excited state of the thyroid gland which is normal for rats.

A comparison of the data obtained by us with that obtained, by Kursanov and co-workers and Gabe and Parrot is of significance. It follows from this comparison that in rats kept under our dietary conditons, i.e., under such conditions that the thyroid gland remains in a quiet state, the catechin preparation exerts no, or almost no, influence on the gland, even in very large doses; when the diet was supplemented with iodized casein (the Biochemical Institute Diet) which increased the functioning of the thyroid gland, the addition of catechin preparation inhibited the hyperfunctioning of the gland which was due to the presence of iodine. According to Gabe and Parrot a hyperfunctioning of the thyroid gland was observed in guinea pigs on a vitamine C- and P-free diet (it is normally in a quiet state); upon the addition of vitamin P to P-avitaminotic animals the functioning of the thyroid gland returned to normal. Therefore, it can be concluded that the catechin preparation normalizes the functioning of the thyroid gland only when the latter is in a state of hyperfunction.

In conclusion we can also note that large doses of catechin preparation (10, 15 mg per rat per day) in our experiments exerted no apparent detrimental influence on the experimental animals.

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THE PHOSPHOSERINE CONTENT AND SPECIFIC ACTIVITY OF PHOSPHORUS IN PHOSPHOPROTEINS OF BRAIN TISSUES

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Of all the known methods for the quantitative determination of phosphoproteins in tissues, the method based on the determination of inorganic phosphorus which splits off upon an 18-20 hours alkaline hydrolysis, as described by Schmidt and Thannhauser [1], is considered the most reliable. Due to the low content of phosphoprotein phosphorus in tissues (of the order of 2-5 mg %) the authors recommend for its precipitation from the hydrolyzate the method of Delory [2] in which a mixture of $CaCl_2$ and $MgCO_3$ is used as the precipitant. With this precipitant it is possible, in a relatively short time (30-40 minutes at 0°), to obtain nearly complete precipitation of small amounts of inorganic phosphorus from dilute solutions. The latter fact is extremely important in experiments with radioactive phosphorus, where the removal of minute amounts of inorganic phosphates, which have no significance in ordinary chemical analysis, is necessary.

The rate of phosphoprotein phosphorus regeneration as well as the exchange of a number of phosphorus compounds in the brain tissue of rats were studied in our previous investigations [3]. The fractionation of phosphorus was performed by the method of Schmidt and Thannhauser, with the precipitation of inorganic phosphorus according to Delory.

On the basis of the data obtained we concluded that the phosphoprotein phosphorus in brain tissue is converted at a quite different rate than, for instance, the phospholipid phosphorus or the main bulk of protein phosphorus. Its degree of regeneration 2 hours after the addition of P^{32} sometimes reached 100% and more. This laid open to question the accuracy of the method applied by us. In order to solve this problem we performed the following experiments.

The experiments were performed on rats as described previously [4]. The magnesium-calcium precipitate containing phosphoprotein phosphorus was dissolved in weak hydrochloric acid and the inorganic phosphorus was then determined by the method of Fiske and Subbarow.

First of all, in order to determine the purity of the phosphorus precipitated by the mixture of $CaCl_2$ and $MgCO_3$, the magnesium-calcium precipitate was incinerated with a mixture of sulfuric and nitric acids, after having been dissolved.

As can be seen from Table 1 the amount of phosphorus in the incinerated samples is significantly higher than in the corresponding samples where the phosphorus was determined without previous mineralization. This testifies to the fact that the residue contains some organic phosphorus in addition to the inorganic phosphorus which formed upon the hydrolysis of phosphoproteins. The amount of the former was found to be of the same order as that of the phosphoprotein phosphorus. The specific activity of the phosphoprotein phosphorus may have been raised due to the presence of this organic phosphorus *.

* The specific activity (SA) is the number of pulses per minute per 1 mg of phosphorus in the investigated fraction.

TABLE 1

Amount of Phosphorus in the Precipitate Obtained by the Delory Method from a Solution of Phosphoproteins, before and after Its Incineration.

(μg per 1 g of brain tissue)

Expt. No.	Without incineration of the residue	After incineration of the residue
1	34	58
2	37	70
3	35	55
4	41	60

In order to determine the extent of the deviation which may be tolerated under such conditions, and in order to determine the true value of the specific activity of phosphoprotein phosphorus, we attempted to separate the organic from the inorganic phosphorus in our samples. For this we followed Berenblum and his co-workers [5], who indicated the possibility of a quantitative extraction by isobutanol of a phosphomolybdenum complex from aqueous solution.

In our experiments, the magnesium-calcium precipitate was dissolved in weak HCl, the molybdate and the color-producing agent were added, and the samples were extracted for 20-30 seconds by isobutanol after the formation of a blue color. The colored product entered the alcohol layer, which was separated by centrifugation and analyzed photometrically. The same isobutyl extracts were then placed on glass slides, dried, and their specific activity determined with the aid of the "B" counting device. In extraction of the phosphomolybdenum complex by isobutanol it is necessary to take into consideration the fact that, depending on the volume ratios of the water and alcohol layers, the volume of the latter will vary and influence the intensity of the coloring. For this reason and in order to obtain accurate quantitative results in the photometric analysis of the samples, it is necessary to establish a specially calibrated curve obtained on the extraction of the samples from a known volume of isobutanol, and to use in subsequent work the same ratios of the aqueous and alcoholic phases. In case of variations in this ratio, the volume of the alcoholic phase must be determined each time and the intensity of the color corrected for the corresponding volume.

The least variations in the volume of the alcoholic phase are observed in the presence of a volume ratio of 3.5-4 alcohol and 5 water. A change of the volume ratio in the direction of an increase in the volume of alcohol leads to an increase in the volume of the alcoholic phase, while a decrease leads to the opposite result.

Preliminary control experiments with standard solutions of labeled inorganic phosphate showed that with such a treatment of the samples an almost total extraction of the phosphorus in the form of a phosphomolybdenum compound actually occurs upon isobutanol extraction. This was demonstrated by the agreement of the SA values of phosphorus in samples before and after isobutanol extraction, and also by the absence of any noticeable radioactivity in the aqueous layer after the removal of the colored isobutanol.

The next necessary step was to determine whether any organic phosphorus compounds, giving no coloration without preliminary incineration but which exhibit radioactivity, are extracted by isobutanol under the conditions of our experiments with tissues. A phosphoprotein phosphorus residue was obtained from brain tissue, the color reaction with the molybdenum reagent performed, the mixture extracted with isobutyl alcohol, and the SA of phosphorus in the isobutyl extract determined before and after its incineration.

TABLE 2

Content and Specific Activity of Phosphorus in the Isobutyl Extract before and after Incineration of the Phosphorus Compounds

Expt. No.	μg of P per 1 g of brain tissue		Specific activity	
	Without incineration	After incineration	Without incineration	After incineration
1	40	33	5250	5000
2	30	30	5000	5000
3	37	34	4730	4860
4	36	33	5000	5100
5	40	33	5190	5300

As seen from Table 2 neither the total content nor the SA of phosphorus in the isobutyl extract changed significantly in the samples that were reduced to ashes. This indicates that under the above described conditions only the inorganic phosphorus is extracted by isobutanol, and that the isobutyl alcohol extraction does not affect the SA of inorganic phosphorus.

In the next series of experiments we compared the SA of phosphoprotein phosphorus extracted in the usual way, i.e., as a magnesium-calcium precipitate with the SA of phosphorus extracted as a phosphomolybdenum compound by isobutanol (Table 3).

TABLE 3

Comparative Data on the Amount of Phosphorus and its Specific Activity in Phosphoproteins of the Calcium-Magnesium Precipitate and of the Phosphomolybdenum Compound Extracted by Isobutanol

Expt. No.	μg P per g brain tissue		Specific activity			Relative specific activity	
	Calcium-magnesium precipitate	Isobutyl extract	Inorganic phosphorus of brain	Calcium-magnesium precipitate	Isobutyl extract	Calcium-magnesium precipitate	Isobutyl extract
1	33	33	14880	15680	4500	>100	30
2	30	32	15730	16700	4330	>100	27
3	35	34	11460	8860	3460	77	30
4	34	34	16500	14770	3530	90	21
5	36	37	12500	11330	3710	92	30
6	34	34	15450	13720	5300	90	34
7	34	33	9100	8490	4320	93	47
8	37	40	13400	9680	5000	72	37
9	35	34	14420	12600	5400	87	37
10	41	34	14400	10170	5380	70	37

* Relative specific activity (RSA) = $\frac{\text{Specific activity of the phosphorus of a given fraction}}{\text{Specific activity of the inorganic phosphorus}} \cdot 100.$

It follows from the above data that, in regard to the amount of phosphorus, we obtained identical results in both cases. The specific activity of the samples after their treatment with isobutanol proved to be 2-2.5 times lower than the activity obtained upon direct measurement of the radioactivity in the solution of the magnesium-calcium precipitate. In the latter case apparently a significant rise of the activity of the samples occurs as a result of the radioactivity of the organic phosphorus which precipitates along with the magnesium-calcium precipitate. As demonstrated by special control experiments, the SA of the organic phosphorus which precipitates along with this residue is quite significant and close to the SA of phosphoprotein phosphorus.

Substitution of a magnesia mixture for the Delory reagent, while somewhat lowering, did not completely eliminate the above contamination. This is demonstrated by the results of one of the control experiments given in Table 4.

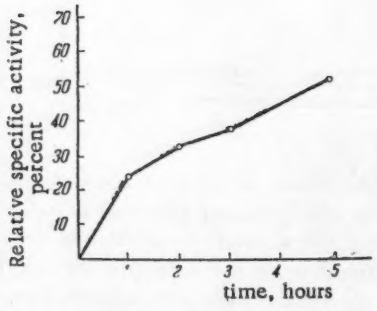
In Samples Nos. 3 and 6 before the addition of the magnesia mixture and after neutralization the main bulk of inorganic salts was removed by centrifugation (see Lisovskaya [6]). The use of the magnesia mixture as the precipitant of inorganic phosphorus is also undesirable for other reasons. First, it extends the time of analysis to 17-19 hours instead of 30 minutes required when the Delory reagent is used; second, even upon a lengthy precipitation of the samples to which the magnesia mixture has been added an incomplete precipitation of the inorganic phosphorus from dilute solutions is observed. In our experiments it reached 30-40%. In the case of determination of phosphoprotein phosphorus this is of great importance since the content of this

form of phosphorus in brain tissue is quite small. Analysis of the phosphorus fraction which remains in solution is also thereby complicated.

TABLE 4

Extent of Inorganic Phosphorus Precipitation and Its Specific Activity Obtained by Various Methods of Precipitation

Sample No.	Method of precipitation	μg P per 1 g of fresh tissue	Non-precipitated phosphorus, in % of samples 1 and 2	SA	RSA
1	According to Delory, 30 minutes, at 0°, phosphomolybdenum blue extracted with isobutanol	42	–	9950	45
2	Same	42	–	9900	45
3	Magnesia mixture, 19 hours at 0°, without isobutanol extraction	27	36	16140	80
4	Same	27	36	14430	66
5	Same	29	31	15600	71
6	Magnesia mixture, 19 hours at 0°, phosphomolybdenum blue extracted with isobutanol	25	40	11050	50
7	Same	27	36	10470	47
8	Same	27	36	9500	43



Variation of the relative specific activity of phosphoprotein phosphorus as a function of time

Therefore, on the basis of all of the obtained data, we came to the conclusion that determination of the true specific activity of phosphoprotein phosphorus upon the precipitation according to Delory is possible only after its extraction by isobutanol in the form of phosphomolybdenum blue.

Using this approach we determined the RSA of phosphoprotein phosphorus at various times after the introduction of P^{32} into animals.

These experiments demonstrated (Figure) that the true RSA of phosphoprotein phosphorus increases with the duration of the experiment and that it reaches 55-60% in experiments which lasted 5 hours.

As we know, phosphoserine is one of the main phosphorus-containing components of such representative phosphoproteins as casein, ovalbumin and others. We attempted to separate the phosphoserine from the phosphoproteins of brain tissue and to compare the SA of the phosphorus entering into the composition of phosphoserine with the SA of phosphoprotein phosphorus.

We used the method of paper chromatography for the separation of phosphoserine. The animals were killed 3 hours after the introduction of radioactive phosphorus. After removal of the acid-soluble and the liquid fractions, the brain tissues from 4-5 rats were treated with a special mixture of solvents (an acid chloroform-methanol mixture) with the aid of which we were able to extract from the protein residue of the tissue all of the phosphorus which we had designated previously [4] as the X-fraction, and which is known in the literature as the "inositic" or the "residual protein phosphorus". The dried tissue residue was extracted with a 10% solution

of NaCl (2 hours at 100°) in order to remove the nucleic acids, then washed with water, alcohol, and ether, and dried. As shown by the results of our analysis, under such treatment of the tissue the phosphorus obtained from the protein residue consisted basically of phosphoprotein phosphorus containing only a small contamination of little-active phosphorus, the SA of which resembled that of ribonucleic acid.

A portion of the powdered tissue, equivalent to 1 g of brain, was hydrolyzed with 10 ml of 1 N NaOH (for 24 hours at 37°) in order to determine the SA of the total phosphoprotein phosphorus. In order to separate the phosphoserine, the remaining portion was partially acid-hydrolyzed with 2 N HCl for 10 hours, according to Lipmann [7]. The small precipitate of phosphoserine obtained from the neutralized hydrolyzate, after precipitation with alcohol and reprecipitation, was dissolved in a small amount of water and applied to the paper for chromatography. The solvent for chromatography was a mixture consisting of n-butyl alcohol, propionic acid and water in the ratio of 23:11:16. Additional chromatograms were developed with ninhydrin and a molybdenum reagent [8]. As a reference spot, we used phosphoserine isolated according to Lipmann [7] and obtained from a preparation of casein which was purified according to Hammarsten.

All of the spots obtained on the chromatograms which were developed with ninhydrin were cut out, minced, and incinerated with a mixture of concentrated H_2SO_4 and HNO_3. Then the phosphorus was determined in the samples after their extraction with a small amount of isobutanol and the activity of the preparations was determined on the same samples.

In all cases, after the ninhydrin development of the chromatograms, pink spots appeared at the level of the reference spot. On the additional chromatograms, developed with the molybdenum reagent, blue spots appeared at the same places 1 to 2 hours after treatment of the chromatograms. In some cases, on the ninhydrin-developed chromatograms, 1 or 2 light pink spots, which usually did not produce any blue spots upon development with the molebdenum reagent, appeared below the main spot. The chromatograms treated with the molebdenum reagent showed compact blue-brown spots immediately after heating and below the main blue spots, which usually did not develop with ninhydrin, and the R_F of which was close to that of the inorganic phosphorus.

As a rule, after the incineration of the bits of paper from the ninhydrin-developed chromatograms, the phosphorus and the radioactivity were detected only in those spots which appeared at the level of the reference spot and which developed with the molybdenum reagent. This identifies this phosphorus as the phosphorus of phosphoserine.

TABLE 5

Rate of Regeneration of Phosphoprotein, and of the Phosphoserine Obtained from Them, in Brain Tissues

Expt. No.	Specific activity of inorganic phosphorus	Phosphoproteins		Phosphoserine	
		SA	RSA	SA	RSA
1	—	9500	—	8500	—
2	20000	9100	45.5	6880	34.4
3	21000	8700	41.4	6800	32.3
4	16700	8100	48.5	6600	39.5
5*	15600	5050	32.3	3200	20.5
6*	22700	8150	35.9	3500	15.4

* In Experiments 5 and 6, the chromatography was performed from the following mixture of solvents: n-butanol – glacial acetic acid – water (8:20:20).

The data on the SA of the phosphorus from the total phosphoproteins and of the phosphoserine isolated from them are given in Table 5. It follows from the given data that the SA of the phosphoserine phosphorus is somewhat lower than that of the total phosphoproteins, but as shown by the data of Table 6 it is significantly higher than the SA of the phosphorus of a number of other phosphorus-containing compounds in brain tissue.

TABLE 6

Comparison of the Rate of Regeneration of the Various Phosphorus Fractions in the Brain (upon a 3 hour exposure)

Type of compound	SA	RSA
Inorganic phosphorus	20 000	100
Lipid phosphorus	464	2.3
Phosphorus of X-fraction including the inositic phosphorus	3466	17.3
Phosphorus of ribonucleic acid	766	3.8
Phosphorus of phosphoproteins	9 100	45.5
Phosphorus of phosphoserine	6880	34.4

The high SA of the isolated phosphoserine indicates that the latter originated from phosphoproteins, since the insignificant amount of organic phosphorus which remained in the alkaline hydrolyzate of the tissue protein residue under the conditions of our experiment exhibits a SA which is almost 10 times smaller than the SA of the phosphoserine phosphorus.

DISCUSSION

The data of some authors [6, 9] who have shown, in vitro, in brain slices, the relationship of the rate of P^{32} incorporation in phosphoproteins to the metabolism of the slices, and also the experimental material presented in this article, convinced us that the phosphoproteins are no artifacts, in spite of the doubts expressed in the literature. In regard to the methods of phosphoprotein determination we can definitely state that the method of Schmidt and Thannhauser [1], while being quite suitable for the quantitative determination of phosphoproteins, is inaccurate in its usual form for the determination of the SA of phosphoprotein phosphorus.

This is the result of the fact that upon the Delory precipitation of inorganic phosphate from the neutralized alkaline hydrolyzates, a significant amount of organic compounds containing radioactive phosphorus which do not produce any color with the molybdate, are simultaneously precipitated. This leads to an increase of the activity of phosphoprotein phosphorus and thereby to a distortion of the SA of this phosphorus fraction. An analogous fact has been also described by Ennor and Rosenberg [10]. While investigating the SA of inorganic phosphorus contained in the acid-soluble fraction they observed its increase due to the addition of organic phosphorus-containing compounds. According to the same authors, the addition of a reagent, which usually increases the precipitation, leads only to an increase in the deviation of the specific activity of inorganic phosphorus when the latter is determined under the conditions of its precipitation from solutions which contain active organic compounds. From our point of view, the most accurate and reliable method for the quantitative determination of phosphoprotein phosphorus and of its SA is the following modification of the method of Schmidt and Thannhauser. After the Delory precipitation of the inorganic phosphorus obtained upon the alkaline hydrolysis of phosphoproteins and the color reaction with the molybdate and the color-producing reagents, the colored solution must be extracted with isobutanol. The phosphomolybdenum blue present in the isobutanol may be used for the quantitative determination of phosphorus by photometric measurements as well as for the determination of the specific activity. The use of the method of isobutanol extraction is convenient also because it makes it possible to concentrate into small volumes samples which are weakly colored. This has great significance in those cases where a small amount of substance is being

analyzed. Similar recommendations for the determination of the SA of inorganic phosphorus precipitated from extracts containing active organic compounds are encountered in the literature.

The previously reported [3] values of the RSA of phosphoprotein phosphorus (on the order of 80-100% per 2 hours) are much too high. As follows from the data reported in the present article, the true value of the RSA of phosphoprotein phosphorus for the same period of time reaches only 30-40%.

The obtained results necessitate a review of our concept of the rate of phosphoprotein conversion in brain tissue.

It is most probable that the data present in the literature in regard to the very high values of phosphoprotein conversion in brain and other tissues, obtained without accounting for the possible fault due to the presence of active organic phosphorus, are not free of such error. For instance, in the work of Skvirskaya and her coworkers [12] the SA of phosphoprotein phosphorus surpassed in a number of cases that of the acid-soluble fraction. According to the data of Lisovskaya [6] the phosphoprotein phosphorus in brain slices in vitro is 58-62% converted after only 60 minutes.

Although it is necessary to correct the previous data, the phosphoproteins remain one of the most active phosphorus fractions of brain tissue among the high-molecular substances.

The isolation of an active phosphoserine from brain phosphoproteins gives an indication of the nature of the active group of phosphoproteins. Analogous data on the isolation of phosphoserine from phosphoproteins of ascitic tumor cells are given by Kennedy and his co-workers [13], and from the phosphoproteins of some rat organs by Agren and his co-workers [14]. In both cases the phosphoserine was isolated by the authors after a partial acid hydrolysis of the tissue protein residue treated according to Schneider. This precipitate contained, in addition to the phosphoprotein phosphorus, the so-called "residual protein phosphorus" or the "inositic phosphorus" designated previously by us as the X-fraction [4]. It was demonstrated [13] that the SA of phosphoserine, isolated from ascitic tumor cells, is higher than the SA of "fresh" phosphoprotein phosphorus by a factor of 4.

In our experiments the SA of phosphoserine phosphorus was somewhat lower than the SA of total phosphoproteins. A possible explanation of this is that the phosphorus in the brain phosphoproteins may be bound to the protein in a different fashion, as it has been demonstrated in casein [15]. Therefore, phosphoserine, while being the main, cannot be considered as the only, phosphorus-containing component of the phosphoprotein molecule. It is then most probable that upon a partial acid hydrolysis some of the phosphorus groups of phosphoproteins which exhibit a higher conversion rate than the phosphorus of phosphoserine may be acid-sensitive and be split in the form of inorganic phosphate, or on the contrary may remain in the form of peptide fragments; in either case we were unable to determine them.

SUMMARY

Determination of phosphoproteins by the method of Schmidt and Thannhauser with the precipitation, according to Delory, of the inorganic phosphorus which forms after hydrolysis, leads to a significant increase of its specific activity. The true specific activity of phosphoprotein phosphorus may be determined by the extraction of the latter, in the form of phosphomolybdenum blue, with isobutanol.

The relative specific activity of rat brain phosphoprotein phosphorus reaches, in 2 hours, 30-40% instead of the 80-100% which was previously determined.

The specific activity of phosphoserine phosphorus isolated from the phosphoproteins of brain tissue is relatively high, and similar to the specific activity of the phosphorus of initial phosphoproteins.

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THE LOCALIZATION OF CYTOCHROME OXIDASE IN THE PLANT CELL

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It can be considered as definitely established that cytochrome oxidase is localized on protoplasmic structures in animals as well as plant cells. Moreover cytochrome oxidase exhibits its catalytic activity only when it is present on the structures. It has been previously demonstrated [1] that all the treatments which disrupt the bond between this enzyme and the structural elements of the cell and which provoke its dilution into the surrounding media, lead to its inactivation. In the animal cell, the cytochrome oxidative system is mostly localized in the mitochondria which apparently carry out a basic function in the respiration of the cell [2, 3].

The problem of the localization of cytochrome oxidase in the plant cell is still open to debate. In early investigations of this problem it has been determined that cytochrome oxidase is localized on structural elements of the cell which are separated from the homogenate of the tissues of various plants by centrifugation at approximately 20,000 g [1, 4, 5]. At such centrifugation speeds, whole and broken plastids are separated. Depending on the conditions prevailing during the separation of these structures and on the material being investigated, some cellular organoids of the type of mitochondria enter this fraction.

Later on, using the method of differential centrifugation, DuBuy and his co-workers [6] succeeded in separating a homogenate of tobacco leaves into fractions of chloroplasts and mitochondria, and in demonstrating that these structures are carriers of cytochrome oxidase activity to identical extents. Contrary to the above, McClendon [7] followed by Jagendorf and Wildmann [8] concluded, on the basis of their experimental results, that the cytochrome oxidase activity was only exhibited by mitochondria.

The doubts expressed by these authors on the localization of cytochrome oxidase in chloroplasts resulted from the absence of any correlation between the distribution of the enzyme activity and the chlorophyll content among the various fractions of the homogenate, and also from their observation that the cytochrome oxidase activity in chloroplasts was lowered by repeated washing of the latter. At the same time, an attempt completely to separate the cytochrome oxidase activity from chloroplasts was unsuccessful [7]. However, cytochemical data show that in the onion epidermis the cytochrome oxidase activity is localized in the microsomes and is absent from the mitochondria and leucoplasts [9]. The contradictory observations present in the literature prompted us to perform some new experiments on the localization of cytochrome oxidase in plant cells.

EXPERIMENTAL

The experimental material was tobacco of the "Trapezond" variety. The plants were grown under field conditions. The leaves were collected from specific nodes before budding, during budding, and during flowering. In order to duplicate the conditions of the experiments of Jagendorf and Wildmann the plants were kept in the shade for 2, 4 and 7 days before the collection of the leaves for the experiment. At the same time we performed a series of experiments on normal, non-shaded leaves. A comparison of the results obtained from fractions of shaded and non-shaded leaves demonstrated their complete identity. For the sake of brevity, we give the results obtained only on non-shaded leaves.

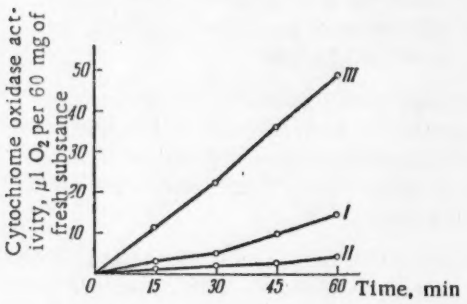
The isolation of chloroplasts was performed in the following manner: 250 g of leaf material was homogenized in a porcelain mortar with 150 ml of a phosphate-sucrose mixture of pH 7.1 (0.4 M sucrose). The leaf homogenate was strained through linen and the obtained fluid was centrifuged at 50 g. The supernatent fluid was decanted and again centrifuged at 550 g for 12 minutes. The supernatent fluid obtained upon centrifugation of the chloroplasts at 550 g was again separated into 2 fractions, which sedimented at 7,000 g for 45 minutes, and at 22,000 g. The latter fraction was obtained on a Sharples centrifuge. The rate of flow of the fluid was 120 ml per minute. Each fraction was washed with the phosphate-sucrose mixture. All of the manipulations were performed at a

temperature of 3°. Upon microscopical observations it was determined that the first fraction, sedimenting at 50 g, contained cell fragments, starch granules, and whole as well as broken chloroplasts. The fractions obtained at 550 and 7,000 g consisted of fragments of plastids. The fraction sedimenting at 22,000 g consisted of a brown residue with a low chlorophyll content. According to electron microscope data this fraction consisted of particles measuring from 0.2 to 4 microns.

The activity of cytochrome oxidase was determined manometrically. The reaction media contained, in final concentrations: 60 mg of dry weight (2 ml) of a suspension of a known fraction of the homogenate; cytochrome C $-2 \cdot 10^{-5}$ M, hydroquinone 1/30 M, and diethyldithiocarbamate (DDC) 1/375 M. The control vessels contained, in one series of experiments, a suspension of the same fraction with all the reagents, including cytochrome C (Control 1), and in another series, all the reagents, including cytochrome C, and a suspension of a known fraction of leaf homogenate (Control 2) previously inactivated by boiling. The central portion of the vessel contained only 0.3 ml of a 20% solution of KOH. The total volume of the vessel was 3.3 ml. The temperature was maintained for 20 minutes. The measurements were taken at 25°. The nitrogen was determined according to the micro-Kjeldahl method. Chlorophyll was determined on the SF-4 spectrophotometer, in alcohol solution, by the absorption coefficient at the absorption maximum of chloroform.

RESULTS

The fraction sedimenting at 550 g was used in the first experiment. This fraction contains the largest amount of chlorophyll (see Table 2). The results of the determinations of the cytochrome oxidase activity in such a fraction of chloroplasts are presented on the Figure.



Absorption of oxygen by chloroplasts in the presence of cytochrome C:
I) Control 1; II) Control 2; III) experiment.

It is apparent from the figure that the addition of cytochrome C to a system containing chloroplasts, hydroquinone and DDC increases the oxygen absorption approximately by a factor of 3 in comparison with the reaction media without the addition of cytochrome C.

Upon determination of the nitrogen of chloroplasts, on the basis of these data on the figure, it was found that the cytochrome oxidase activity per 1 hour consists of 9.13 µl of O_2 per 1 mg of nitrogen. In other experiments this fraction reached 44.7 µl of O_2, i.e., an activity twice as high as that calculated by Jagendorf and Wildmann [8] for the same fraction. It may be assumed that the high activity of this fraction which we observed is the result of its contamination with mitochondria which according to the above authors exhibit the main cytochrome oxidase activity. In order to eliminate any possible contamination of the chloroplasts, this fraction was washed 5 times with a phosphate-sucrose mixture (0.4 M sucrose, phosphate buffer 1/15 M of pH 7.1). The results of a number of such experiments are given in Table 1.

TABLE 1

Cytochrome Oxidase Activity as Influenced by the Washing of Chloroplasts

(The activity is expressed in µl of O_2 per 1 mg of N per 60 minutes)

Experimental fraction	Treatment of plastids	Date of experiments					
		7/10	7/15	7/18	7/26	7/28	8/2
Fraction of chloroplasts, obtained at 550 g for 12 minutes	Without washing	9.1	13.3	18.4	23.8	22.2	44.7
	Washed 5 times with a sucrose-phosphate solution	53.5	56.3	68.1	62.7	91.26	79.2

In all experiments, the leaves were taken from the same nodes [8-10] before the beginning of budding Variations in the absolute activity are apparently the result of ontogenetical variations of cytochrome oxidase. Contrary to the results obtained by other authors [7, 8], a repeated washing of the chloroplasts, or the elimination of contamination due to small structural units, leads not to a decrease in the cytochrome oxidase activity, but to its significant increase.

The 3-4 fold increase of cytochrome oxidase activity after the washing of chloroplasts is difficult to explain only by the removal from this fraction of particles which do not exhibit any cytochrome oxidase activity. Such an effect is most probably due to the removal by washing of some substances which inhibit the cytochrome oxidase system. As shown by subsequent experiments these substances may be the products of the oxidation of polyphenols, which form intensively upon the destruction of the cell.

Determination of the function of individual structures in any process necessitates a comparative study of the enzyme activities of various organoids. For this purpose the homogenate was separated into various fractions. Each fraction was twice washed with a phosphate-sucrose mixture, and its enzyme activity determined.

TABLE 2

Cytochrome Oxidase Activity in Various Fractions of Tobacco Leaf Homogenates

Fraction	Duration of centrifugation (minutes)	Relative centrifugal force (g)	Content of chlorophyll				Cytochrome oxidase activity, in μl of O_2 per 60 minutes					
			In % dry substance		In mg per 1 mg N		Per 100 mg fresh substance		Per 100 mg dry substance		Per 1 mg N	
			Experiment I	Experiment II	Experiment I	Experiment II	Experiment I	Experiment II	Experiment I	Experiment II	Experiment I	Experiment II
Residue	1	50	1.66	1.87	0.35	0.41	92.2	142.8	294.6	465.6	72.5	99.4
	5	50	4.58	3.35	0.73	0.75	107.2	225.3	530.8	795.4	85.1	155.6
	12	550	4.66	4.42	0.77	0.90	226.3	347.0	1019.3	1397.0	191.7	186.1
	45	7000	2.86	2.53	0.56	0.53	131.6	143.3	434.5	417.4	88.8	58.8
Centrifugate		22,000	0.66	0.69	0.08	0.14	85.2	117.7	358.7	482.3	45.8	83.1
			1.17	1.20	–	1.39	0.0	0.0	0.0	0.0	0.0	0.0

As can be seen from Table 2, all fractions of the homogenates from tobacco leaves contain chlorophyll to some extent. Such a distribution of chlorophyll is apparently related to the conditions under which the structures are separated and in the first place to the careful homogenization of the tissue, which leads to the braking of the chloroplasts into fragments of various sizes. At the same time the chlorophyll is unequally distributed among these fractions. Its highest content is observed in the fraction which sediments at 550° g. Contrary to the data obtained by Jagendorf and Wildmann, it is this fraction which exhibits the highest cytochrome oxidase activity; moreover, such a characteristic holds whether the calculations of the enzyme activity are made on the basis of the dry weight, the fresh weight, or the nitrogen. In the remaining fractions a correlation between the content of chlorophyll and the cytochrome oxidase activity is observed. The supernatent fluid remaining upon the sedimentation at 22,000 g is an exception. Apparently the absence of any cytochrome oxidase activity in this fraction, in spite of the presence of a small number of chloroplast fragments, is due to the presence of specific inhibitors.

What, then, is the reason for the discrepancy between our data and those of McClendon[7], Jagendorf and Wildmann [8]? One of the possible reasons for this contradiction may be related to the different state of the investigated chloroplasts, which resulted from some differences in the conditions under which these structures were isolated in our experiments and in those of Jagendorf and Wildmann.

It follows from the results obtained by the above authors that the cytochrome oxidase activity is the highest in that fraction of the homogenate which contains the greatest amount of disrupted chloroplasts. Possibly, a high cytochrome oxidase activity in the chloroplast fraction results from the violent disruption of

whole chloroplasts in the process of their ejection from the cell, apparently contributing to a better expression of the cytochrome oxidase activity.

Another possible reason for the divergence of the results obtained upon the investigation of cytochrome oxidase in structural elements of the plant cell may lie in the various methods used for the determination of cytochrome oxidase. Jagendorf and Wildmann determined cytochrome oxidase without accounting for the action of polyphenol oxidase. However, it is known that along with cytochrome oxidase a polyphenol oxidase with an apparent specificity of hydroquinone is actively functional in tobacco leaves [10].

The data on the distribution of polyphenol oxidase in the plant cell are also contradictory. Bonner and Wildmann [11] found polyphenol oxidase only in the cytoplasm. Sisakyan and Kuvaeva [12] have found it in leucoplasts, Arnon [13] points to the presence of this oxidizing system in chloroplasts, while Ducet and Rosenberg [5] have found it in chloroplasts as well as in the cytoplasm.

In order to exclude the activity of polyphenol oxidase in our experiments, we always added DDC to the reaction media for the determination of the cytochrome oxidase activity. However, preliminary experiments demonstrated that upon the addition of DDC to the system containing chloroplasts, cytochrome C, and hydroquinone, instead of the expected decrease in oxygen absorption resulting from the inhibition of polyphenol oxidase an increase in oxygen absorption was observed. The hypothesis of the oxidation of DDC by the suspension of chloroplasts, similarly to the oxidation of DDC by animal tissues [14], was not confirmed.

The increase in oxygen absorption upon the addition of DDC to the system apparently results from the fact that DDC, inhibiting polyphenol oxidase, hinders to some extent the accumulation of the products of the oxidation of polyphenols, which inhibit the activity of cytochrome oxidase.

The probability of the hypothesis of the inhibition of cytochrome oxidase by the products of the oxidation of polyphenols is substantiated by the fact that quinone exerts a negative influence on oxygen absorption by chloroplasts in the presence of cytochrome C. It is apparent from Table 3 that the addition of quinone to the system decreases the oxygen absorption by 40-70%.

TABLE 3

The Influence of DDC and Quinone on the Cytochrome Oxidase Activity of Chloroplasts*

Date of experiments	Reaction media					
	Chloroplasts + hydroquinone + + cytochrome C + DDC		Chloroplasts + hydroquinone + + cytochrome C		Chloroplasts + + hydroquinone + + cytochrome + + DDC + quinone	
	μl of O_2	in %	μl of O_2	in %	μl in O_2	in %
9/9	96.6	100	73.6	77.1	58.3	60.9
9/14	193.8	100	117.3	60.5	103.4	53.5
9/16	133.5	100	57.3	50.4	30.6	23.0

* Final concentration of quinone: 1/30 M.

Taking into consideration the fact that Jagendorf and Wildmann [8] determined the cytochrome oxidase without taking into consideration the simultaneous oxidative process due to the activity of polyphenol oxidase, which inevitably leads to the accumulation of the products of polyphenol oxidation, it may be granted that one of the possible reasons for the divergence of the obtained results lies in the different conditions under which the activity of cytochrome oxidase was determined.

SUMMARY

An active cytochrome oxidase is functional in isolated chloroplasts of tobacco leaves. Repeated washings of the fraction of chloroplasts by a phosphate-sucrose mixture leads to a significant increase in the activity of cytochrome oxidase.

Cytochrome oxidase activity was detected in all the fractions of a homogenate from tobacco leaves, with the exception of the supernatent fluid obtained by centrifugation at 22,000 g. The highest cytochrome oxidase activity is exhibited by the fraction of chloroplasts which sediments at 550 g. The same fraction contains the greatest amount of chlorophyll.

The activity of cytochrome oxidase must be determined in the presence of DDC, thus preventing the accumulation of quinones, which inhibit cytochrome oxidase.

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REMEMBRANCES OF PROFESSOR S. M. PROKOSHEV

November 7, 1955 marked the death of Professor and Doctor of Biological Sciences, Sergei Mikhailovich Prokoshev, renowned specialist and scientist who directed the laboratory of the A. N. Bakh Institute of Biochemistry of the Academy of Sciences of the USSR.

S. M. Prokoshev was born on July 4, 1905 in Kadnikov of the Vologodsk district. In 1928 he graduated Leningrad University and went to work at the All-Union Institute of Horticulture where he successfully enlarged his scientific and public activity until 1940. In 1936 he presented a dissertation for the degree of Candidate of Biological Sciences.

In 1940 he went to obtain his doctorate at the A. N. Bakh Institute of Biochemistry of the Academy of Sciences of the USSR. In 1944 he presented his doctoral dissertation and then occupied the position of Senior Fellow in Science. On August 1, 1950 he was appointed director of the Laboratory of Secondary-Synthesis Substances.

S. M. Prokoshev, who completed his studies at an excellent school of plant physiology and biochemistry, creatively used his knowledge and experience and devoted all his efforts and ability to the solution of important practical problems in biochemistry. He was one of the first investigators in the field of accelerating the ripening of fruits, and, in particular, of tomatoes under the influence of ethylene. He showed that a stimulating effect may be obtained also during brief action of this gas on green fruits, which was verified and widely used in the works of other authors.

The investigations of S. M. Prokoshev on the biochemistry of the potato constituted an important chapter in our science. He investigated various sorts and types of potato for contents of starch, protein and vitamin C, determined the most valuable of these, which served as the basis for selection works. The investigations of S. M. Prokoshev on the study of the mechanism of the reactions of injured plant tissues, the mechanism of ascorbic acid formation and its role in plants are of interest.

The monograph of S. M. Prokoshev "Biochemistry of the Potato", which was his doctoral dissertation, achieved wide renown and is the only monograph on this theme in world literature. The potato as a food product, its chemical composition and variability and the physiological aspects of storing potatoes were treated at length and from many viewpoints. Sergei Mikhailovich devoted much attention to the investigation of potato in connection with its resistance to the Colorado beetle. He and his associates carried out a broad study on plants of the potato family for their content of glycoalkaloids. It was shown that the resistance of the potato to the beetle does not always depend on the presence of demissin but involves the specific nature of the exchange and enzymatic functions of the given types of potato.

S. M. Prokoshev was deeply interested in the formation and transformation of organic acids in plants, believing that organic acids are the link in the exchange of organism that interconnects various facets of the exchange of substances.

He obtained exceptionally valuable results in investigating new sources of raw material for the preparation of organic acids, as the result of which he established that the leaves of the cotton plant are very rich in citric and malic acids and can satisfy the needs of industry for organic acids. He recently undertook the investigation of the problem of potato degeneration, the solution of which is a current problem for potato culture in the south of our nation. He published more than forty scientific works.

S. M. Prokoshev was an able specialist in the biochemistry of plants, possessed a broad scope and a sharp mind, thanks to which he always was capable of actively participating in the solution of important biological problems.

S. M. Prokoshev actively participated in scientific-social life. For a long period of time he successfully fulfilled his obligations as chairman of the Commission for Fighting the Colorado Beetle; he was the member of a commission of experts on biology for the Higher Certification Commission, member of scientific comm-

ittees of the Institute of Biochemistry and the Institute of Plant Physiology of the Acad. Sci. USSR. A characteristic trait of S. M. Prokoshev was his sincerity and principle. He worked on the chief problems and left his associates much latitude for independent research.

The untimely death of S. M. Prokoshev is a great loss for us, the coworkers of his laboratory, the Institute and for biological science and agricultural science.

G. S. Ilyin

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